Conference on the Use of BCG in Therapy of Cancer

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FRANK J. RAUSCHER, JR., *Director, National Cancer Institute*

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CONFERENCE ON THE USE OF BCG IN THERAPY OF CANCER

Held at
National Institutes of Health
October 5 and 6, 1972

Edited by:
Tibor Borsos
and
Herbert J. Rapp

With the technical assistance of:
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OPENING REMARKS

I was the one who sent out all the letters inviting you; if anything goes wrong with the meeting from an administrative standpoint, you have me to blame. If everything goes right, you have to thank Mrs. Nancy Branch, my secretary, who has done an enormous job to get everybody here.

I would also like to extend special thanks to Dr. C. Gordon Zubrod for his encouragement and help.

This meeting is a result of a conversation between Dr. Frank Rauscher and Dr. Herbert J. Rapp at a Christmas party, December 1971. I am sorry that the ultimate date of this meeting came when some unfortunate publicity concerning bacillus Calmette-Guérin (BCG) has reached the national-international press. It was not planned this way.

I will ask the press to reserve their questions for the intermissions or until after the meeting.

Dr. William D. Terry will summarize this 2-day meeting tomorrow afternoon.

Tibor Borsos
Biology Branch
National Cancer Institute
Bethesda, Maryland 20014

WELCOMING REMARKS

Ladies and gentlemen, on behalf of my colleagues here at the National Cancer Institute, welcome and thank you for coming. As some of you know, I have recently gotten a new job. I am still amazed to find myself in this position. As I told many people, including the President, I didn't really want this job, and now, after about 5 months, I find that my judgment was excellent.

One of the nice things about the position is that I can perform the delightful duty of welcoming groups such as this. We have had many meetings, of course, in recent years here at the National Cancer Institute. I can think of none that had the potential of this meeting. I think this is partly because, in some circumstances, there is the possibility of direct application to the patient and perhaps even to the prospective patient. This is exceedingly important.

This meeting has to do with this issue of accountability that is so much in the eyes and thoughts of the President and of the Congress. I have promised them on many occasions in our testimony and in our reviews by the Office of Management and Budget that our scientists in this country and throughout the world are highly motivated to do whatever can be done now with existing information to benefit the patient and the prospective patient. I have also promised that we will be accountable for the generous influx of new funds this year and hopefully in the years to come.

This meeting also has to do with overexpectancy on the part of the lay public and the Congress. A concurrent issue here is that of overpromise. I urge those friends from the press to be sure in their accounts of this meeting that we do not convey overpromise, and therefore overexpectancy on the part of the public.

By the end of this meeting we will have a fairly good feel for the status of the phenomena about which you're talking and a feel for what can be done more rapidly with the new resources that have been provided. What is really needed to develop the information you have now, to test it? Can the work be done better? Can it be done sooner if additional resources were provided for this kind of work? Then, finally, what resources are necessary?

Thank you.

Frank J. Rauscher, Jr.
Director
National Cancer Institute
Bethesda, Maryland 20014

INTRODUCTORY REMARKS

It is indeed a pleasure and honor for me to open this meeting on the use of BCG in the therapy of cancer.

It is safe to say that immunotherapy of cancer has reached that critical mass of knowledge and available expertise which are necessary for the quantum jump. Many have said—and I think it's not without some degree of accuracy—that chemotherapy led the way and that immunotherapy and immunology are about where chemotherapy was some 15 years ago. However, there are some people who will argue with this statement.

A major thrust of the new cancer act is cancer control. And as part of cancer control, and as Dr. Frank Rauscher has stressed on many occasions, delivery of what we can now do for the cancer patient is extremely important.

For example, if we could reduce the cancer mortality in the United States, site for site, to that of the country reporting the lowest mortality, we would save approximately 100,000 lives a year in the United States.

Cancer deaths could be reduced 90% within the present century if, in addition to current knowledge on prevention and universal application of the best diagnosis and treatment techniques, our research could uncover: 1) the environmental elements causing migrants' cancer rates to change toward those of their new countries; 2) the environmental elements that lead to different rates in different countries throughout the world; and 3) of most importance, the susceptible individuals whom we can and must protect.

We now have information to reach these goals for cancer of the lung and cancer of the uterine cervix and corpus. Prevention of these types of cancer alone would account for 60,000 lives a year saved in this country.

How do we best implement what we can now do? Do we do it through education, demonstration clinics, or subsidy, which has been mentioned? This may be a saving in the long run in certain types of cancer—e.g., the demonstrated relationship between lung cancer and the carcinogenicity of tobacco smoke. Although "control" can mean many things, certainly it includes early diagnosis and treatment.

The prospect for widespread application of immunotherapy is ripe today. Whether it be precisely in therapy, immunodiagnosis, or immunoprophylaxis, certainly the work of the people who have convened here today is of utmost importance to the attainment of these goals. Your work can be applied to all three of these areas, as well as to the understanding of the basic mechanisms of the cancer-host relationship.

An indication of the present importance of immunology is evidenced by the development of an organizational locus for immunology at the National Cancer Institute. As you know, this is, at least for patient therapy, under Dr. William Terry in the Division of Cancer Biology and Diagnosis.

Exactly how the other aspects of immunology, including immunotherapy, will be structured remains to be worked out, but at least I think it is moving and moving well.

At the risk of not including everybody, I would like to mention a few people who are intimately concerned with this, people who will be speaking to you and to whom you will be talking in the next few days: Dr. Herbert J. Rapp, Dr. Tibor Borsos, and Dr. Berton Zbar in the carcinogenesis area, which is in the Division of Cancer Cause and Prevention; Dr. Charles Boone, Dr. Michael Chirigos, and Dr. Paul Levine in the viral oncology area (also in the Division of Cancer Cause and Prevention) ; Dr. Mary Fink and Dr. William Hammond in the Division of Cancer Grants, who are concerned with the grant applications for these studies; Dr. Paul Carbone, Dr. Ronald Herberman, and Dr. Brigid

J. Leventhal in the Division of Cancer Biology and Diagnosis; and finally Dr. Seymour Perry in the Division of Cancer Treatment.

All of these people and many others are dedicated and committed to the advancement of knowledge in this exciting area of cancer control and cancer research. It is therefore a very appropriate time for this meeting, and I know it will be an interesting and productive one.

Again, let me say welcome. Thank you.

James A. Peters
Director, Division of Cancer
Cause and Prevention
National Cancer Institute
Bethesda, Maryland 20014

ANIMAL MODELS

Discussion Leaders: George B. Mackaness (Morning Session) and
David W. Weiss (Afternoon Session)

INTRODUCTION: ANIMAL MODELS OF IMMUNOTHERAPY OF CANCER

According to Helen C. Nauts, for over 200 years physicians have observed and recorded dramatic "spontaneous" regressions of various types of neoplastic disease. One factor that seemed to be associated with these spontaneous regressions was concurrent acute bacterial infection. This is probably why W. B. Coley, who was Helen Nauts' father, began his studies on the treatment of human cancer by the administration of toxic products of bacterial metabolism known collectively as "Coley's toxin."

Coley's work on this subject is documented in a series of more than 100 papers published between 1891 and 1936. A striking observation recorded by Coley was that results were best when the infection occurred in, or the toxin was administered directly on, the tumor. This principle of Coley will be referred to at least twice during this Conference: first when Berton Zbar talks about our studies on the guinea pig model which were started 10 years ago at the National Cancer Institute and second when Edmund Klein talks about his remarkable work at the Roswell Park Memorial Institute on human cancer. I also suspect that the data to be presented by some of the other speakers may be interpretable in the light of Coley's principle.

The first tumor-immunity experiments with animals were reported in 1904. In terms of tumor immunology, however, the work done by Coley was more scientifically sound than that being done with animals. In the human studies, clinicians were dealing with primary autochthonous tumors, while the animal experimenters were using transplantable tumors not genetically compatible with the recipients. When it was generally recognized that so-called experimental tumor immunologists were actually establishing the basis for transplantation immunology, the field collapsed and in the wake of its destruction went all the years of effort by Coley and other clinicians who had recognized the possible importance of immunology in the treatment of cancer. These clinicians were powerless to avert the disaster because not only was there now no experimental basis for their approach, but also their own work was dismissed as the work of charlatans.

Tumor immunology in the 1930's and 1940's, therefore, was definitely moribund, and all investigators with even a shred of sanity had given up the patient for lost. One brave soul, Ludwik Gross, however, dared to publish a paper in 1943 in which he claimed that tumors induced by the administration of 3-methylcholanthrene in genetically identical mice could be used to immunize other mice of the same strain so that a subsequent challenge with an ordinarily lethal dose of tumor cells was rejected. In this publication, Gross single handedly established the basis of modern tumor immunology and outlined many of the important generalities of the discipline. These generalities have been rediscovered from time to time and still are being rediscovered by the growing army of investigators who now call themselves "tumor immunologists."

Most studies in tumor immunology have been a variation on the theme composed by Gross; i.e., they are model studies on the prevention rather than the treatment of cancer by immunologic methods. Indeed, the first demonstration in an autochthonous model that BCG might be an effective anticancer agent was that of Lloyd Old and coworkers who showed more than 10 years ago, with a prevention model, that the rate of appearance of tumors was decreased in animals treated with BCG during the administration of a chemical carcinogen.

Reviewing tumor immunology a few years ago, Old indicated that no immunologic maneuver had ever eliminated an established tumor. One of our goals in 1963, when I

started feeding diethylnitrosamine to guinea pigs, was to devise a model suitable for testing the question of whether the immune system can reverse the growth of established tumors. Berton Zbar is going to tell you how we accomplished this and, in the bargain, how we found special conditions under which not only the primary transplant can be eliminated but also cells that have spread to the draining lymph node.

Where do we go from here with our animal models? We now have to develop models that will permit us to approach the clinical situation as closely as possible. Thus we need to study cancer immunotherapy in animals with primary autochthonous metastatic cancer. The work of Richard Simmons, about which you will be hearing this morning, represents an important start in this direction.

In the meantime, I think enough is now known to try to make a logical plan of attack on human cancer. This plan must be guided by information derived from scientifically sound animal studies. What do these animal model studies mean for human cancer? What should be done in the clinic? What should not be done in the clinic? Let me preface the answers to these questions by saying that, if we are lucky, BCG treatment may be useful in a limited number of different neoplasms. It is not a panacea.

First, cancer immunotherapy with BCG should not be attempted in advanced or terminal cancer. The obvious corollary of this is that, if BCG is used clinically, it should be used only in those cases where metastases are first detected and have not spread much beyond the draining lymph node.

Second, BCG should be administered in such a way as to promote intimate contact between the primary cancer and the BCG.

Third, BCG therapy should be started before surgery, radiation, or chemotherapy. The scientific basis for these recommendations should become evident from some of the data to be presented this morning.

These data tell me that it is time to stop thinking of immunotherapy as an adjunct to conventional modes of cancer treatment. The propagation of this cliché will prolong the time until it is known whether BCG therapy will be of value in the clinic. These data also tell me that it is time to stop saying that: "BCG has already been tried in the clinic and it doesn't work." The propagation of this cliché will also prolong the time until it is known whether BCG therapy will be of value in the clinic. These data tell me it is time to give cancer immunotherapy a real chance to show what it might do before the delicate balance of the immune system is disturbed by conventional modes of therapy.

Most of us who have been privileged to work with Frank Rauscher from time to time receive memoranda from him that end in the words with which I would like to use to start this Conference: "Let's get on with the job."

Herbert J. Rapp
Biology Branch
National Cancer Institute
Bethesda, Maryland 20014

An Experimental Model for Immunotherapy of Cancer[1]

Berton Zbar, Edgar Ribi,[2] and Herbert J. Rapp, *Biology Branch, National Cancer Institute,*[3] *Bethesda, Maryland 20014*

SUMMARY—The biologic features of an animal immunotherapy model are described. Requirements for successful therapy are: limited tumor size, injection of BCG into the tumor site, and a host capable of developing and expressing an immune reaction of the delayed-hypersensitivity type to mycobacterial antigens. The tumor-suppressive properties of a nonliving BCG preparation are described.—Natl Cancer Inst Monogr 39:3–6, 1973.

WE HAVE developed an animal model for immunotherapy of cancer. In this model, immune therapy causes regression of established tumors and prevents the growth of lymph node metastases (*1, 2*). Inbred guinea pigs receive intradermal injections containing 10^6 syngeneic tumor cells. Six days after injection, a 100-mg tumor nodule develops at the site of inoculation; treatment is instituted at this time. One group of guinea pigs is treated by intralesional injection of living *Mycobacterium bovis* (strain BCG), a second group by intralesional injection of diluent, and a third group by excision of the tumor nodule. The result of treatment is shown in figures 1A–C and 2 and in table 1: All animals treated by intralesional injection of diluent develop progressively growing lymph node metastases and die; all animals treated by excision of the tumor nodule are free of tumor at the site of excision but develop progressively growing lymph node metastases and die. Surgery fails because tumor cells are present in draining lymph nodes at the time of excision. Intralesional injection of BCG causes complete regression of established intradermal tumors and prevents the growth of lymph node metastases. Of the guinea pigs with 100-mg tumors, 58% are alive and free of tumor 1 year after treatment; of the guinea pigs with 500-mg tumors, 16% are alive and free of tumor 1 year after treatment.

In addition to limited tumor size, there are 2 other requirements in this model for successful

TABLE 1.—Treatment of intradermal tumors in syngeneic guinea pigs by intralesional injection of living BCG*

Day of treatment	Number of animals	Percent animals alive and tumor free 1 year after treatment
6–7	51	58
12	23	16

* Summary of 8 experiments performed at the National Cancer Institute between July 1970 and October 1972. All guinea pigs treated by intralesional injection of saline or by excision of the intradermal tumor died from progressive tumor growth.

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] *Present address:* Rocky Mountain Laboratory, Hamilton, Mont. 59840.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

therapy. 1) BCG must be injected into the tumor. Intradermal injection of BCG adjacent to, or contralateral to, tumor is ineffective treatment (table 2), as is intradermal injection of BCG before tumor inoculation (*3*). 2) The host must be able to develop and express an immune reaction of the delayed-hypersensitivity type. Procedures that impair this type of immune function impair BCG immunotherapy. For example, treatment with rabbit anti-guinea pig lymphocyte serum impairs development of delayed hypersensitivity to mycobacterial antigens and abrogates BCG-mediated tumor killing (*4*). Intravenous injection of large numbers of living BCG organisms impairs development of delayed hypersensitivity to mycobacterial antigens and abrogates BCG-mediated tumor killing (*3*).

We have recently described a nonliving mycobacterial preparation with potent tumor-suppressive properties (*5*): the cell walls of BCG attached to oil droplets (fig. 3). Preparations containing the cell walls alone or cell walls not attached to oil droplets are ineffective. Of the guinea pigs treated by intralesional injection of BCG cell walls attached to oil droplets, 59% are alive and tumor free 80 days after treatment (table 3). The effective dose range is 30–300 μg of cell walls admixed with 1.4 μl oil.

TABLE 2.—Comparison of ability of BCG located at site of tumor inoculation, contralateral to site of tumor inoculation, and adjacent to site of tumor inoculation to suppress tumor growth

Group	Injected with line-10 tumor cells + BCG	Size of tumor nodule at day 22 (radius squared, mm²)	
1	Same site*	7±1	*P*†—
2	Separate contralateral sites	150±13	<0.01
3	Separate adjacent sites	149±39	<0.01

* 1.5×10^6 line-10 tumor cells and 6×10^6 living BCG organisms were injected intradermally into the same site or into separate sites. The results represent the mean value ± SE mean of 5 animals. The incidence of intradermal tumors in group 1 was 0/5; in group 2, 5/5; and in group 3, 5/5.

† Tumor growth in groups 2 and 3 is compared to that in group 1.

TABLE 3.—Treatment of intradermal tumors in syngeneic guinea pigs by intralesional injection of cell walls of BCG attached to oil droplets*

Day of treatment	Number of animals	Percent animals alive and tumor free 80 days after treatment
6–8	53	58

* Summary of 6 immunotherapy experiments performed between September 1971 and October 1972. All guinea pigs treated by intralesional injection of Tween saline or oil droplets died from progressive tumor growth.

In collaboration with our laboratory, this model has been used by Dr. G. B. Mackaness to study the tumor-suppressive potency of different BCG substrains, by Dr. Ribi to define molecules of the tubercle bacillus responsible for tumor suppression, and by Dr. M. G. Hanna, Jr., to define histopathologic features of BCG-mediated tumor killing.

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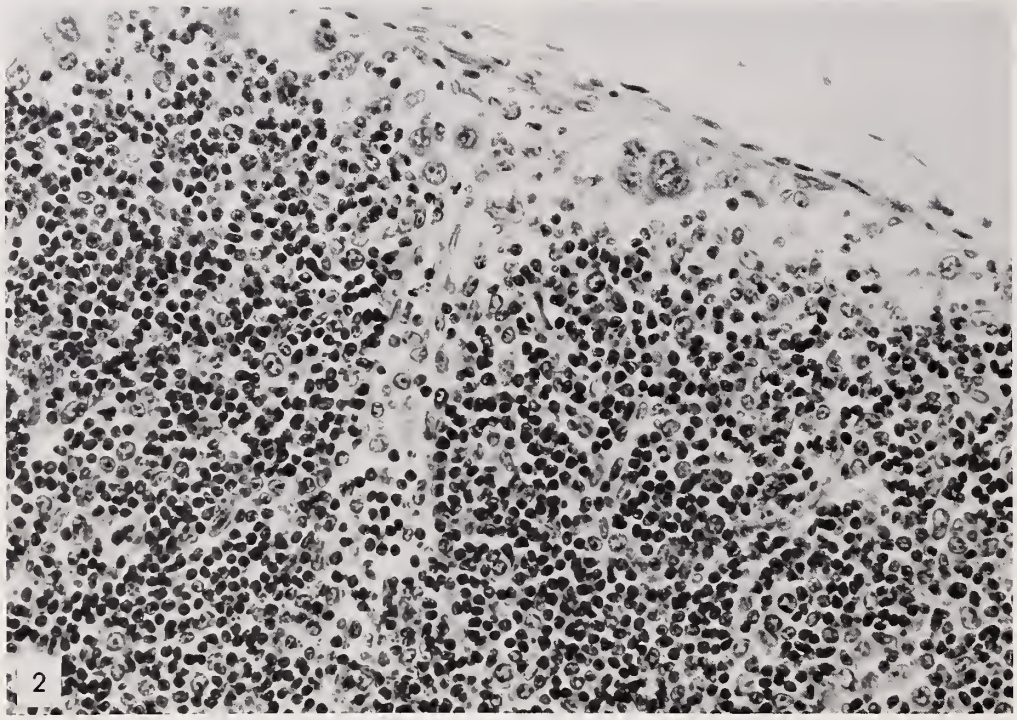
(*4*) HANNA MG, JR, SNODGRASS MJ, ZBAR B, et al: Histopathology of tumor regression after intralesional injection of *Mycobacterium bovis*. IV. Development of immunity to tumor cells and BCG. J Natl Cancer Inst 51:1897–1908, 1973

(*5*) ZBAR B, RAPP HJ, RIBI EE: Tumor suppression by cell walls of *Mycobacterium bovis* attached to oil droplets. J Natl Cancer Inst 48:831–835, 1972

FIGURE 1A.—Guinea pig treated by intralesional injection of diluent 6 days after intradermal injection of 10^6 line-10 tumor cells. Photograph was taken 38 days after injection of tumor cells. *Note* intradermal tumor (posterior) and enlarged superficial distal axillary lymph node (anterior). Animal died 66 days after injection of tumor cells.

FIGURE 1B.—Guinea pig treated by local tumor excision 6 days after intradermal injection of 10^6 line-10 tumor cells. Photograph was taken 38 days after intradermal injection of tumor cells. *Note* absence of intradermal tumor and the presence of enlarged superficial distal axillary lymph node. Animal died 89 days after injection of tumor cells.

FIGURE 1C.—Guinea pig treated by intralesional injection of living BCG 6 days after intradermal injection of 10^6 tumor cells. Photograph was taken 38 days after intradermal injection of BCG; *note* vertical scar at the site of intratumor injection of BCG; enlarged superficial distal axillary lymph node is absent. Animal was alive 1 year after injection of tumor cells.



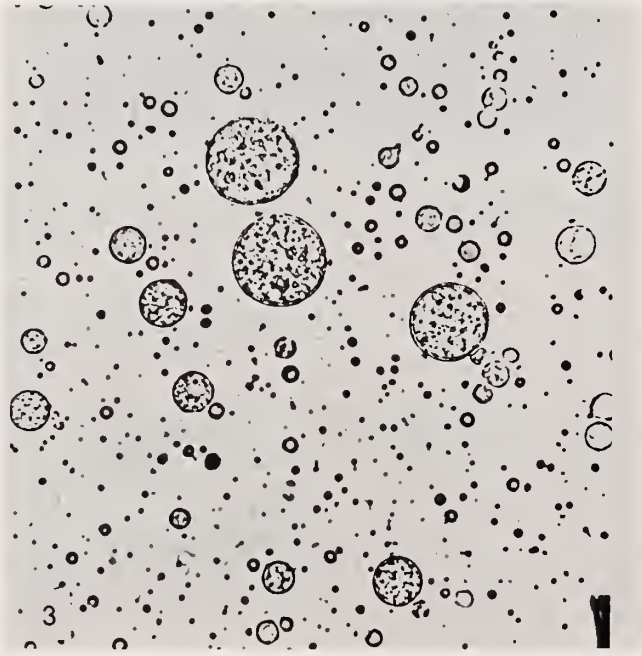


FIGURE 2.—Lymph node draining intradermal tumor. Guinea pigs were given intradermal injections of 10^6 line-10 tumor cells. They were killed 7 days after tumor cell injection, and complete autopsies were performed. Superficial distal axillary node, first lymph node draining the tumor site, is shown. *Note* tumor cells in subcapsular space. Tumor cells are not in cortex.

FIGURE 3.—Photomicrograph of preparation of cell walls of BCG attached to oil droplets. *Note* oil droplets vary in size from 1–2μ to 10–20μ. Opaque material, bacterial cell walls are attached to oil droplets.

DISCUSSION

G. B. Mackaness: Are there any questions of a technical nature?

M. Chase: Have you ever used guinea pigs presensitized to mycobacteria?

B. Zbar: Yes. The results are not significantly different.

C. Vogel: You mentioned that thymectomy and antilymphocyte serum abrogated the response of intralesional BCG. The situation is analogous to malignant melanoma in man. Have you looked at the effect of lymphadenectomy?

Zbar: I'm not sure I understand your question. If you remove the local tumor and the draining lymph node—this was done by Dr. Hanna—the animals still succumb to progressive disease, indicating that the tumor cells are beyond the draining node and the tumor site. Are you asking whether lymphadenectomy influences the immune response to the bacillus?

Vogel: Yes.

Zbar: We haven't tested that.

J. L. Ziegler: Have you used any tumors other than transplanted tumors?

Zbar: In the guinea pig we have used only transplanted tumors. Dr. Bartlett has done some experiments in primary 3-methylcholanthrene-induced tumors in mice that he perhaps could discuss later.

W. D. Terry: This is a further amplification of one of the preceding questions; then I will ask one other question. If not only the immediate draining node is removed but also at the same time the regional node is dissected—i.e., would one try to take out the draining chain as one might in a surgical situation—what happens therapeutically in that situation? In the same context, one might ask the question about chemotherapy administered at the same time.

Zbar: These experiments have not been done.

Terry: I think in the discussion we will want to come back to that. It's a minor point and quite a different point from the major one which needs to be established and about which we are talking: Does immunologic manipulation allow one to destroy an established tumor? Another point, which becomes more important when we talk clinically, is how does that manipulation stack up against other comparable forms of therapy presently available? The specific question then is: If the tumor is not intradermal, but subcutaneous or intramuscular, what happens when one injects BCG into the tumor in the guinea pig model?

Zbar: An experiment was done once in which the tumor was inoculated intramuscularly, and BCG was subsequently inoculated into the same site. In this situation, BCG administration was ineffective.

L. Chedid: How much oil was used? Was your preparation comparable to Freund's adjuvant?

Zbar: It should be stressed that the material developed by Dr. Ribi is extremely different from Freund's adjuvant. The only thing these materials have in common is that they both have oil and cell walls. The material Dr. Ribi prepares has a minute amount of oil. The amount of oil injected into the tumor is 0.7 of a microliter, with the amount of cell walls varying from 30 to 300 μg.

Chedid: Could I follow-up on that by asking you whether the first data were obtained with live BCG?

Zbar: Yes.

Chedid: Did you try killed BCG under similar conditions with oil emulsion or droplets? Is the cell wall preparation crude or purified?

Zbar: We have not mixed heat-killed tubercle bacilli with oil and injected this mixture.

Regarding the last question, the cell walls are what are referred to as "crude cell walls." They have not been treated with proteolytic enzyme or anything of that sort. The cell walls by themselves do not work. It is important also to note that a preparation in which the cell walls and oil droplets are in the same mixture but not physically attached also does not work. The cell wall must be attached to the oil droplet to get the antitumor effect.

S. R. Rosenthal: You mentioned that, when you give BCG other than intratumor proper, the tumor progresses. Does it progress as much when you give nothing at all? You mentioned 145 mg. What is the weight when you give no injection of any kind?

Zbar: As far as I can tell, there's no difference.

J. E. Sokal: I assume that your cell wall material induces tuberculin sensitivity.

Zbar: Yes.

Sokal: Is it as potent to the living organism in sensitizing the guinea pig?

Zbar: This hasn't been tested rigorously, but it seems to be. For example, if you test them with a standard dose of purified protein derivative (PPD), they react at the same threshold dose.

P. Alexander: Do the live BCG organisms proliferate in the draining node? Are they just filtered out by the draining node, and do they also appear in the subsequent chain of nodes? In other words, is the effect seen only if the tumor-bearing area actually has BCG in it or dividing organisms in it?

Zbar: One answer to the question is that the Ribi cell wall vaccine works in this model, and obviously that doesn't divide.

But the living bacteria are distributed in the draining nodes. If they can be observed histologically, they can be cultured from the lymph nodes and from the spleen of these animals. As to whether they divide in the tissue, I'm sure Dr. Mackaness can answer that better than I can.

Alexander: But then the treatment isn't really systemic; it is really local. You inject the organisms locally into the skin lesion, and they, then, by the organic drainage mechanism, automatically finish up in the draining nodes, so that you are really having a direct injection of organisms into all the successfully treated sites.

Zbar: Yes.

A. Goldin: With advanced tumors or even with earlier

tumors, will surgical excision of the tumor and then use of the BCG increase the response?

Zbar: No.

C. M. Pinsky: I have 2 questions, Dr. Zbar. 1) Getting back to what Dr. Rosenthal asked, could you say anything about the dosage used, the number of viable organisms, the number to cause your effect, and the number to cause the progression if you inject it elsewhere? 2) Have you given injections to the draining node as opposed to the tumor when they are both still there?

Zbar: The number of bacteria that make the injection effective has been worked out recently by Dr. Mackaness: about 2×10^7 living organisms per injection site.

We have not done the experiment you suggest, i.e., injecting the bacteria directly into the draining lymph node without giving the intradermal tumor an injection.

D. Windhorst: In relation to that same question, what about the dose of organisms in the immunologically compromised nodes? Have you had a chance to look at that?

Zbar: A dose of bacteria that will completely regress the tumor in the uncompromised animal does not work in the immunologically compromised mouse or guinea pig.

Windhortst: I'm getting at whether you use less organisms.

Zbar Are you asking whether the bacteria are going to kill the animal when it is immunologically compromised? They have not in the guinea pig and the mouse; these animals have not died from systemic injection.

M. J. Mastrangelo: I'd like to get back to Dr. Rosenthal's question regarding the intramuscularly transplanted tumor. It was my impression from the data presented at the recent meeting of the American Association for Cancer Research that, when the intramuscular tumor was given an injection of BCG, it did not regress but, in fact, progressed more rapidly than had it not been given the injection. Is that not correct?

Zbar: It's not a good idea to dwell too much on the intramuscular experiments because they have not been done in as much detail as the experiments to which I alluded. If you'd like to discuss these experiments perhaps a little later, that would be better than now because there are not that many experiments and I don't want to generalize on the basis of those experiments.

J. W. Thomas: Could I refer to the question of presensitization and ask you if your comments apply to the fact that you notice no difference irrespective of the size, i.e., the 6-day tumor and the 12-day tumor? Did desensitization improve the 12-day results?

Zbar: Direct comparisons were not made so I can't definitely answer your question.

What we have observed is a consistent tendency for the unimmunized animals to respond slightly better than immunized animals. When we did dose-response tests in unimmunized and immunized animals, the dose of bacteria required to inhibit tumor was somewhat less in an unimmunized animal than it was in an immunized animal.

So although the specific question you asked has not been tested experimentally, everything suggests that being unimmunized at the start of one of these tumor experiments is to the animals' advantage.

Sokal: Have you tried sensitizing the animal with BCG, making him strongly tuberculin positive, and then using intralesional injection of PPD instead of either the cell wall or the living bacteria?

Zbar: That is an exceedingly important point. Actually, most of our original studies were done in this way. We found the tumor was only partially suppressed when sensitized animals received injections of tumor cells mingled with PPD. The tumor was completely suppressed when sensitized or immunized animals received injections of tumor cells and living BCG. These experiments indicated that a chronic inflammatory reaction of the delayed-hypersensitivity type was essential for tumor suppression. A 48-hour reaction elicited by PPD was insufficient.

Dr. Klein will have more to say about what he found when he did this kind of experiment clinically. But in the animal model, PPD gives only transient tumor inhibition in a sensitized animal. But again, this is a single injection of PPD as opposed to the more repeated injections that Dr. Klein has given.

Sokal: But there is some effect?

Zbar: Yes, but transient.

Ziegler: From a clinical viewpoint, it would be very interesting to analyze the nonresponders, the 40% guinea pigs who failed to be cured in this experiment. At some point we have to ask ourselves why this doesn't work in all the animals. The mycobacteria produce polymorphic lesions, depending, I presume, on host factors such as in leprosy. Could the BCG lesion that you produce in the responders versus the nonresponders differ in histologic appearance?

Zbar: That's a good point. It hasn't been looked at carefully.

H. J. Rapp: We don't know about the nonresponders yet because we haven't reached the top of the dose-response curve. I wouldn't use the term "nonresponders" because they all had prolonged life as a result of BCG treatment. If we use a higher dose of BCG, we might increase the number of total cures.

B. H. Waksman: Isn't the crucial issue whether any of the tumor cells have left the local site and the local node? If you have any single tumor cell that has migrated elsewhere in the body and is not in contact with the tubercle bacilli, then this is going to be without effect. Do you agree?

Zbar: We know experimentally that the tumor cells are in the first draining node and in other nodes, but in the same chain. We do not know for certain that there are tumor cells more distant from these sites. This brings up the other point, i.e., that these animals eventually develop systemic tumor immunity which can kill tumor cells without the need for close contact with the tubercle bacillus. So that there are at least two things going on: 1) what results from the local BCG response and 2) the development of specific tumor immunity which can kill distant small numbers of tumor cells.

Mackaness: Have you thought to short-circuit the first node by injecting BCG not only into the lesion but also

beyond in order to have access to the nodes beyond the primary node?

Zbar: That's a good suggestion.

J. C. Kennedy: In relationship to this same point, do you get BCG in the draining nodes of the presensitized animals or does presensitization prevent local dissemination of the BCG?

Zbar: We haven't tested this.

Mackaness: There is certainly a difference in the dissemination beyond the first node. BCG has access to the first draining nodes but it probably will not get beyond the presensitized animal.

W. W. Shingleton: You described what I interpret to be a tumor-specific immune event. What data do you have on humoral or cellular immunity in the animals that respond and in those that don't?

Zbar: We have become convinced over the last year that the in vitro tests we have used give little information as to the outcome of one of these experiments, and we have also been impressed that we can't make any correlations of a prognostic type from any of the in vitro tests we have used.

C. G. Zubrod: It would be nice to see the evidence about the remote effects, because without it, all you have is another form of local treatment equivalent to surgery plus removal of lymph nodes, or surgery plus radiation of the lymph node-bearing area.

Mackaness: Dr. Zbar, what happens if you irradiate the tumor mass when it is too large to be treated by this procedure?

Zbar: This is actually being studied now. We don't have any results yet.

R. B. Herberman: Dr. Zbar, what in vitro tests have you done to follow cellular immunity? This is an important model for what's being tried in the clinic. Also, have you tried to correlate prognostically the results of skin tests with tumor extracts with those of BCG treatment?

Zbar: The tests that have been used have been lymphocyte transformation, macrophage migration inhibition; there have been some tests of antibody in the serum and a few experiments done for delayed cutaneous hypersensitivity in these animals.

The data are not good enough to discuss at length; they have been discouraging.

D. W. Weiss: When you say you've used lymphocyte transformation, do you mean lymphocyte-lymphocyte reactions as a general indication of immunity or lymphocyte transformation in the presence of the tumor cells?

Zbar: Lymphocyte transformation in the presence of tumor cells. But we are perhaps getting a little bit misled. We have used these tests in tumor-bearing guinea pigs with these tumors over the past year. These tests have been extremely difficult to employ and they haven't given us too much information that we didn't have from the in vivo experiments. We haven't done enough critical experiments in which we injected BCG into the tumor and looked for animals who were cured and not cured to make a very hard statement. It has just been discouraging using these in vitro tests in the tumor-bearing animals.

Mackaness: Thank you, Dr. Zbar, for a stimulating and interesting presentation.

BCG Immunotherapy of Rat Tumors of Defined Immunogenicity[1, 2]

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SUMMARY—Growth of syngeneic transplants of 3-methylcholanthrene (MCA)-induced rat sarcomas was suppressed if the tumor cells were injected together with BCG. Tests were made to determine the optimum dose of BCG and the maximum numbers of tumor cells rejected. Rejection of these inocula suppressed growth of a simultaneous challenge with the same tumor at another subcutaneous site, and the conditions for immunotherapy were determined in terms of maximum tumor-cell challenge and time of treatment. Comparative studies also showed that irradiated tumor cells, in conjunction with BCG, were less effective for immunotherapy than viable cells. Similar methods were evaluated for the treatment of pulmonary tumor deposits produced by intravenous (iv) injection of MCA-induced sarcoma cells. Lung tumor growth was inhibited by specific immunostimulation with viable tumor cells admixed with BCG; but therapy was more effective with iv administration of BCG alone, when given up to 7 days after tumor challenge. Lung tumor growth was also suppressed by iv injection of BCG cell walls on oil emulsion. However, the effectiveness of BCG induced suppression of lung tumors depended on the immunogenicity of the target tumor, since other, more weakly immunogenic tumors were enhanced rather than suppressed.—Natl Cancer Inst Monogr 39: 11–17, 1973.

GROWTH OF syngeneic transplants of diethylnitrosamine-induced guinea pig hepatomas (*1*) and 3-methylcholanthrene (MCA)-induced sarcomas in rats (*2*) and mice (*3*) is suppressed when tumor cells are injected in admixture with BCG. Furthermore, deliberate infection of established local tumors may lead to their rejection (*2, 4*) and, with guinea pig hepatomas (*4*), to the inhibition of lymph node metastases. Direct contact between tumor cells and BCG seems to be an essential prerequisite for good tumor inhibition, and specific responses to tumor-associated antigens have been observed following such rejection (*1, 2*). At this stage, however, it is not known whether tumor-specific immune reactions initiate BCG-mediated inhibition of tumor growth or whether they arise as a consequence of rejection. The present investigation 1) explored the conditions of tumor immunogenicity

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and tumor burden under which BCG is effective for immunotherapy of local tumors, and 2) from these findings, studied the use of BCG for the treatment of pulmonary tumor deposits produced by intravenous (iv) transfer of tumor cells. While it is recognized that this method of producing tumor growth in lungs may differ from the process of spontaneous pulmonary metastasis, it has allowed comparative studies with tumors of defined immunogenicities.

MATERIALS AND METHODS

Tumors.—Tumors were induced with chemical carcinogens or arose spontaneously in rats of an inbred Wistar strain. Each tumor was carried by subcutaneous (sc) transplantation in syngeneic rats of the same sex as the primary donor. Sarcomas Mc7, Mc40A, and Mc52A, induced by sc injection of MCA, were highly immunogenic; rats treated by excision of sc growths rejected a subsequent challenge with whole tumor grafts. Sarcoma Sp24 arose spontaneously and was weakly antigenic; graft-immunized rats rejected a maximum of 10^3 viable tumor cells. Mammary carcinoma AAF57, induced by oral administration of *N*-hydroxy-2-acetylaminofluorene, lacked significant antigenicity; graft-immunized rats failed to reject a challenge inoculum of 5×10^4 cells. Epithelioma Sp1, a spontaneously arising tumor, was weakly immunogenic; immunized rats rejected a maximum of 5×10^4 tumor cells.

Single cell suspensions of tumors were prepared by trypsin digestion of finely minced tissue and resuspended in medium 199. Pulmonary tumor growth was produced by iv injection of single cell suspensions into a lateral tail vein.

BCG.—Freeze-dried viable BCG vaccine (percutaneous), supplied by Glaxo Research Ltd., Greenford, Middlesex, England, was reconstituted in water to 10 mg moist weight of organisms/ml, and further dilutions were made in Hanks' balanced salt solution.

Methods of treatment.—To assess the influence of localized BCG infection on the growth of sarcoma Mc7, defined numbers of tumor cells were mixed with known amounts of BCG and immediately injected sc. Active immunotherapy of sc challenge inocula of sarcoma Mc7 was given by injection at a contralateral sc site of viable, or ^{60}Co γ-irradiated (15,000 R), tumor cells admixed with BCG.

Active immunotherapy of iv transferred tumor cells was given by single or repeated sc injections of cells of the same tumor prevented from growth by admixture with BCG. Treatment was also effected by single or repeated iv injections of BCG.

Assessment of pulmonary tumor growth.—Pulmonary growth of iv transferred tumor cells was demonstated by perfusion of lungs with diluted India ink (*5*). Macroscopic nodules on the lung surface were counted, >200 being scored as 200+. When we examined the influence of immunotherapy on survival following iv injection of tumor cells, animals were killed individually when distressed by pulmonary tumor growth and survival was calculated with respect to the day of tumor cell injection.

RESULTS

Influence of Localized BCG Infection on Growth of sc Transplants of MCA-Induced Sarcomas

Previously we showed that sc transplants of MCA-induced rat sarcomas can be suppressed by direct contact between tumor cells and BCG organisms (*2*). One of these sarcomas, Mc7, was used to define conditions under which maximum inhibition of tumor growth can be achieved in this manner. The minimum effective dose of BCG (moist weight of a standard Glaxo percutaneous vaccine) was initially determined against a fixed challenge dose (6×10^5 cells) of sarcoma Mc7. These tests established that tumor growth could be completely suppressed by the injection of tumor cells admixed with as little as 10 μg BCG (table 1, expt. 1), but consistent results required a minimum dose of 100 μg BCG. When we investigated the maximum tumor cell inoculum rejected when injected sc with a standard dose (200 μg) of BCG vaccine (table 1, expt. 4), we observed complete suppression of tumor

TABLE 1.—Inhibition of sc growth of sarcoma Mc7 cells in contact with BCG

Experiment No.	Mixed cell inoculum: Number of tumor cells	Mixed cell inoculum: BCG moist weight (μg)	Tumor takes: Test	Tumor takes: Control
1	6×10^5	2	2/4	
		5	2/4	
		10	0/4	4/4
		20	3/4	
		100	0/4	
2	5×10^5	100	0/4	4/4
		500	0/4	
3	5×10^5	100	0/4	4/4
4	5×10^5	200	0/17	17/17
	1×10^6	200	0/12	12/12
	2×10^6	200	0/4	4/5
	5×10^6	200	3/8	8/8
	1×10^7	200	2/4	2/2
	5×10^7	200	5/5	5/5

growth from challenge with up to 2×10^6 sarcoma Mc7 cells and partial inhibition with 5×10^6 to 1×10^7 cells.

Immunotherapy of Sarcoma Mc7

Implantation of viable sarcoma Mc7 cells admixed with BCG so that progressive tumor growth is inhibited also prevents growth of a simultaneous contralateral challenge with the same tumor (2). The effectiveness of this treatment was, therefore, compared to that of treatment with heavily irradiated (15,000 R) cells. These experiments (table 2) show that, whereas a standard inoculum of 2×10^6 viable sarcoma Mc7 cells together with 500 μg BCG completely inhibited a simultaneous contralateral challenge with 1×10^6 sarcoma Mc7 cells, tumor growth was only partially suppressed when irradiated tumor cells were used for immunotherapy. We then tested viable sarcoma Mc7 cells mixed with BCG to determine the maximum tumor challenge which could be controlled by this immunotherapy and the time course of the response (table 3). Challenges with up to 1×10^6 sarcoma Mc7 cells were rejected in all but 1 rat receiving a simultaneous contralateral injection of tumor cells and BCG. Tumor growth was only retarded with challenges of 2.5×10^6 and 5×10^6 sarcoma Mc7 cells, and all these rats developed progressively growing tumors. In further tests, we studied the effect of delaying treatment of challenges of either 5×10^5 or 1×10^6 sarcoma cells. At the lower challenge dose (table 3, expt. 3), tumor growth was partially suppressed even when treatment was delayed for

TABLE 2.—Immunotherapy of sarcoma Mc7 at an sc site with BCG admixed with viable or irradiated sarcoma Mc7 cells

Tumor challenge cells	Treatment*				Tumor growth	
	Day	Inoculum (cells)	Cell dose	BCG moist weight (μg)	Treated rats	Controls
1×10^6	0	Viable	2×10^6	500	0/4	4/5
1×10^6	0	Viable	2×10^6	500	0/4	5/5
1×10^6	0	Irradiated	2×10^6	500	2/4	4/4
1×10^6	0	Irradiated	5×10^6	500	2/4	3/5

* Tumor cells in admixture with BCG injected contralaterally to tumor challenge. No tumor growth was observed at this site.

TABLE 3.—Conditions for the immunotherapy of sarcoma Mc7 with viable tumor cells prevented from growth by admixture with BCG

Experiment No.	Tumor challenge cells	Contralateral inoculum			Tumor growth	
		Day	Tumor cell dose	BCG moist weight (μg)	Treated rats	Controls
1	2×10^5	0	1×10^6	200	0/4	4/4
	5×10^5	0	1×10^6	200	0/4	3/4
	1×10^6	0	1×10^6	200	1/4	4/4
2	1×10^6	0	2×10^6	500	0/4	5/5
	2.5×10^6	0	2×10^6	500	4/4*	5/5
	5×10^6	0	2×10^6	500	4/4	4/4
3	5×10^5	0	2×10^6	500	0/4	
		2	2×10^6	500	1/4	4/4
		4	2×10^6	500	2/4	
		7	2×10^6	500	4/4	
4	1×10^6	0	2×10^6	500	0/4	
		2	2×10^6	500	2/4	4/5
		4	2×10^6	500	3/4	
		7	2×10^6	500	3/4	

* Growth retarded in all treated rats.

up to 4 days after challenge; but by 7 days, treatment was ineffective. At the higher cell dose (table 3, expt. 4), significant suppression by a contralateral injection of sarcoma Mc7 cells with BCG was not obtained when treatment was delayed beyond 2 days after challenge.

BCG Immunotherapy of Pulmonary Growths From iv Transferred Rat Tumor Cells

Using techniques developed in tests on the inhibition of local tumor growth, we evaluated immunotherapeutic methods incorporating BCG as adjuvant for the treatment of pulmonary metastases. Pulmonary tumors were obtained by the iv injection of rat tumor cells. While it is recognized that this mode of producing pulmonary tumor growth may differ from the natural metastatic spread of tumors, the technique provides a method for comparing the efficacy of BCG therapy against tumors of defined immunogenicities, under conditions where the time of tumor dissemination and the tumor burden can be precisely controlled.

Active Immunotherapy

Immunotherapy of MCA-induced sarcoma Mc40A injected iv was initially evaluated by response to a simultaneous sc injection of viable tumor cells admixed with BCG (table 4). In 2 experiments, control rats, after showing respiratory distress, were killed at days 14 and 19, respectively; all but 1 rat had multiple (200+) lung tumor nodules. In comparison, only 2 of 9 rats receiving Mc40A cells sc with BCG at the time tumor cells were injected iv had developed pulmonary tumor nodules when the experiments were terminated. In other experiments, rats treated 3 times by immunostimulation with Mc40A cells with BCG survived for 33–54 days (mean 44), whereas mean survival for controls was 30 days. Treated rats also had a reduced number of lung tumor nodules.

BCG Therapy

Tests were also done to evaluate whether pulmonary growth of iv transferred sarcoma cells could be inhibited by iv injection of BCG alone, since this should lead to direct contact with tumor cells in the lungs (table 5). In 2 experiments with sarcoma Mc40A, terminated after 14 and 20 days, respectively, all but 1 of the controls developed extensive pulmonary tumor growths. Rats receiving BCG iv with the sarcoma cells had no detectable tumor nodules when the tests were terminated; tumor growth was also prevented when BCG alone was administered iv 5 or 6 days after tumor cells. In another 2 experiments (table 5, expts. 3, 4), we examined the effect of single or repeated iv injections of BCG on the survival of rats given iv challenge with sarcoma Mc40A. The mean survival in controls was 25 and 30 days, respectively, and all of these rats had multiple lung tumor nodules. A single injection of BCG at the time tumor cells were administered markedly prolonged survival: all rats were alive at termination of the experiment (100 days), and of these, only 2 had detectable lung tumor nodules.

TABLE 4.—Treatment of iv transferred sarcoma Mc40A cells by sc injection of tumor cells mixed with BCG

Experiment No.	Tumor challenge (iv)	Day	Treatment inoculum (sc)*	Mean survival	Number of rats with lung tumors	Lung nodules/rat
1	5×10^6	—	—	Terminated day 14	5/5	$5\times200+$
	5×10^6	0	Mc40A cells, 5×10^6: BCG, 1.5 mg	Terminated day 14	1/5	$1\times200+$
2	5×10^5	—	—	Terminated day 19	4/5	$4\times200+$
	5×10^5	0	Mc40A cells, 3×10^6: BCG, 1.0 mg	Terminated day 19	1/4	70
3	5×10^5	—	—	30 days	4/4	$116,3\times200+$
	5×10^5	0, 5, 10	Mc40A cells, 2×10^6: BCG, 1.0 mg	44 days	4/4	45,72,120,200+

* No tumor growth occurred at sc site of injection of sarcoma Mc40A cells together with BCG.

TABLE 5.—BCG treatment of iv transferred rat sarcoma cells

Experiment No.	Sarcoma	Tumor challenge (iv)	BCG day	Treatment dose, iv (mg moist weight)	Mean survival	Number of rats with lung tumors	Lung nodules/rat
1	Mc40A	5×10^6	—	—	Terminated day 14	5/5	5×200+
		5×10^6	0	1.0		0/5	—
2	Mc40A	5×10^5	—	—		9/10	40,140,7×200+
		5×10^5	0	1.0	Terminated day 20	0/10	—
		5×10^5	5	1.0		0/5	—
		5×10^5	6	1.0		0/5	—
3	Mc40A	5×10^5	—	—	25 days	4/4	130,3×200+
		5×10^5	0	1.0	Terminated day 100	2/5	1,2
		5×10^5	6,10,12,15	4×1.0	78 days	0/4	—
4	Mc40A	5×10^5	—	—	30 days	4/4	116,3×200
		5×10^5	0,3,5,7	4×1.0	75 days	4/5	2,6,10,30
5	Mc7	2×10^6	—	—		4/4	40,140,2×200+
		2×10^6	0	1.0	Terminated day 14	0/4	—
		2×10^6	7	1.0		0/4	—
6	Mc52A	1×10^6	—	—	Terminated day 21	4/4	3,3×200+
		1×10^6	0	1.0		0/4	—
		1×10^6	6	1.0		0/5	—

A comparable increase in survival time and reduction in number of pulmonary tumor nodules were observed when 4 doses of BCG were administered at 2- to 3-day intervals, starting when tumor cells were administered or 6 days later (table 5, expts. 3, 4). In comparable tests with 2 other MCA-induced sarcomas (Mc7, Mc52A), treatment of iv injected tumor cells by iv BCG, either simultaneously or after 6 or 7 days, completely prevented development of pulmonary tumor growth up to the time control rats with generally extensive tumor growth in the lungs had to be killed (table 5).

Table 6 summarizes our preliminary studies on the use of iv BCG cell wall in oil emulsions for the treatment of pulmonary growth of sarcomas Mc7 and Mc52A. These preparations were provided by Dr. B. Zbar of the National Cancer Institute, Bethesda, Maryland, and the formulations were prepared as described by Zbar et al. (*6*). In the test with sarcoma Mc52A, an emulsion of oil alone slightly inhibited the development of pulmonary tumors. Treatment with BCG cell wall (150 μg) in oil emulsion, however, completely arrested tumor growth. In the second experiment with sarcoma Mc7, the cell wall suspension or oil emulsion alone had a tumor-inhibitory effect and markedly reduced the number of pulmonary nodules. The reduction in the number of lung tumor nodules was greater, however, in rats treated with BCG cell walls in oil emulsion.

TABLE 6.—Treatment of iv transferred sarcoma cells with BCG cell walls attached to oil droplets

Tumor	Tumor challenge (iv)	Treatment, iv (day 0)	Number of rats with lung tumors	Lung nodules/rat
Mc52A	3×10^6	—	5/6	5×200+
	3×10^6	Saline in oil emulsion	3/5	3×200+
	3×10^6	BCG cell wall (150 μg) in oil emulsion	0/6	—
Mc7	2.5×10^6	—	5/5	122,128,148,2×200+
	2.5×10^6	Saline in oil emulsion	5/6	1,2,37,100,150
	2.5×10^5	BCG cell wall (150 μg)	6/6	2,16,30,35,36,43
	2.5×10^6	BCG cell wall (150 μg) in oil emulsion	4/6	1,4,12,50

In the experiments so far described, MCA-induced sarcomas, all of which have appreciable immunogenicities, were used to evaluate the effectiveness of BCG and related products for adjuvant therapy of pulmonary tumor deposits. We made further tests using 3 rat tumors with weaker immunogenicities to evaluate whether their pulmonary growth could also be suppressed by BCG. In these tests, tumor cells injected iv in admixture with BCG did not inhibit growth of tumors (table 7). On the contrary, pulmonary growth of all 3 tumors was greatly enhanced by BCG treatment, and increased numbers of lung nodules resulted.

TABLE 7.—BCG treatment of iv transferred rat tumor cells

Tumor	Tumor challenge (iv)	BCG day	Treatment dose iv (mg moist weight)	Mean survival	Number of rats with lung tumors	Lung nodules/rat
Mammary carcinoma AAF57	1×10^5	—	—	Terminated day 22	6/6	9,33,35,42,71,72
	1×10^5	0	1.0		6/6	6×200+
	1×10^5	0,4,8	3×1.0		5/5	5×200+
Epithelioma Spl	1×10^5	—	—	49 days	5/6	3,5,6,7,18
	1×10^5	0	1.0	33 days	6/6	26,30,35,37,54,55
	1×10^5	0,3,7	3×1.0	41 days	6/6	11,18,21,35,50,58
Sarcoma Sp24	1×10^4	—	—	43 days	5/5	3,3,6,7,14
	1×10^4	0	1.0	32 days	5/5	12,22,27,33,38

DISCUSSION

Previously we established that growth of syngeneic transplants of MCA-induced rat sarcoma cells can be suppressed by sc injection in admixture with viable BCG organisms (*2*). The present studies with one of the sarcomas (Mc7) explored the optimum conditions for BCG-mediated suppression of local tumor growth. In these studies a dose of at least 100 μg moist weight of a standard (Glaxo percutaneous) vaccine was necessary to inhibit growth of a tumor cell inoculum (6×10^5 cells) which grows progressively in control rats. The maximum tumor cell inoculum consistently rejected when injected together with BCG was 2×10^6 cells, approximately 10 times the threshold dose for consistent tumor growth. This, therefore, defined the maximum dose of viable sarcoma Mc7 cells which can be used in admixture with BCG for active immunotherapy of this tumor. Quantitation of the immunotherapeutic response of tumor Mc7 further established that a challenge of up to 1×10^6 sarcoma cells can be eliminated completely by a simultaneous contralateral injection of sarcoma Mc7 cells in admixture with BCG. Immunotherapy with viable sarcoma cells prevented from growth by contact with BCG was also effective when treatment was initiated up to 4 days after tumor challenge, although this depended on the size of the initial tumor cell inoculum. Irradiated tumor cells were less effective than viable tumor cells for immunotherapy with BCG. In immunoprotection studies with sarcoma Mc7, in which rats were immunized by repeated implantation of irradiated tumor or by excision of viable tumor grafts, the maximum tumor challenge rejected was 5×10^6–10^7 cells. In this system, therefore, immunotherapy after tumor challenge cannot fully activate the immunologic capacity of the host.

Active immunotherapy by sc injection of viable sarcoma cells in admixture with BCG also inhibited growth of pulmonary deposits of an immunogenic rat sarcoma (Mc40A) produced by iv injection of tumor cells. A more marked inhibitory effect against this tumor and other immunogenic MCA-induced sarcomas in lungs was obtained when BCG was injected iv. Thus pulmonary tumor growth could be almost completely suppressed even when BCG treatment was delayed until 7 days after the initial tumor cell challenge. Since it is well established that iv injected mycobacteria show preferential survival in lungs (*7*), this method of treatment should allow direct contact between BCG orga-

nisms and tumor cells. This condition has already been found essential for suppression of sc growth of these tumors (*2*). Suppression of lung tumor growth by iv BCG also depended on the immunogenicity of the target tumor. Whereas lung tumor growth of strongly immunogenic MCA-induced sarcomas was prevented by iv BCG injection, treatment was ineffective with 3 weakly immunogenic tumors (mammary carcinoma AAF57, epithelioma Sp1, and sarcoma Sp24). On the contrary, BCG treatment of rats inoculated with these tumors enhanced tumor growth, increasing numbers of pulmonary nodules and decreasing survival. These observations support the view that a major component of BCG-mediated tumor suppression is a stimulation of the specific immune response to tumor-associated antigens. This may be initiated by nonspecific responses associated with the strong, granulomatous reaction produced in lungs by BCG (*8, 9*), although the significance of granuloma formation in relation to tumor suppression is unresolved (*10*).

One objective of these studies was to establish experimental animal tumors for screening BCG products for the treatment of both local and disseminated tumors. Using these systems, for example, one can compare subcellular fractions of mycobacteria. In preliminary studies, BCG cell wall preparations on oil droplets inhibited the development of pulmonary growth of MCA-induced sarcomas. These findings agree with the report that BCG cell walls attached to oil droplets inhibit growth of syngeneic guinea-pig hepatoma transplants and provide a basis for the evaluation of other mycobacterial fractions such as cord factor and water-soluble fractions (*6, 11*).

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DISCUSSION

R. T. Prehn: Dr. Baldwin's paper raises questions about which I'd like to go back and ask Dr. Rapp's group. These questions concern the antigenicity of the tumors in the guinea pigs. In particular, how many different independent tumors have been looked at in the guinea pig model, and do these tumors have a range of antigenicities that corresponds in any way to the results achieved?

B. Zbar: We have not looked at a spectrum of tumors in the guinea pig as Dr. Baldwin has done in the rat.

P. Alexander: You have clearly shown that the highly antigenic tumors lodged in the lung could be treated with intravenous injection of BCG. Now, of course, highly antigenic tumors would presumably never give rise to spontaneous hematogenous spread. Is there a correlation? In fact, don't those tumors in which you have deliberately lodged the cells in the lung and which you can treat with BCG metastasize spontaneously? Whereas, do those tumors which you cannot treat belong to the class which would metastasize spontaneously? For example, if you implanted one of your other tumors which didn't respond into the leg and amputated the

leg, would that animal then die of lung metastases? Are only those tumor cells responding that wouldn't metastasize?

R. W. Baldwin: In the rat tumors studied, there is no correlation between their capacity to metastasize and their immunogenicity. For example, a weakly immunogenic fibrosarcoma of spontaneous origin does not metastasize any more readily than the immunogenic 3-methylcholanthrene (MCA)-induced sarcomas. In this respect, we believe that metastasis is not simply controlled by host immunosurveillance. It is relevant, however, to refer to other studies we are carrying out with another transplanted tumor, epithelioma Sp1. This tumor is highly invasive so that even after surgical removal of 4-day-old tumor grafts the rats will die of lung metastases. Furthermore, the tumor is only weakly immunogenic and so may be a good model for human cancer. In this case, BCG infection of local tumor partially suppresses tumor growth and inhibits pulmonary metastasis.

Also, if one surgically excises a subcutaneous tumor after 9 or 10 days and gives BCG intravenously, survival is significantly increased. Comparing these studies with tests uing highly immunogenic tumors such as MCA-induced sarcomas draws attention to the need of defining the immunogenicity of the tumor being treated with BCG.

T. Borsos: Does it matter in your system whether the animals are preimmunized to BCG?

Baldwin: No.

W. D. Terry: I just want to push Dr. Alexander's question a little further in terms of what is known about the antigenicity in general of spontaneous tumors as opposed to those we select out or that we have induced by a variety of chemical carcinogens or viruses. Can we make any generalization about experimentally definable antigenicity or immunogenicity in these categories of tumors?

Baldwin: We looked at the immunology of 2 comparable classes. We looked at spontaneously arising and chemically induced mammary carcinomas in the rat. Both these classes of tumors are comparable in their immunogenicity, but both are less antigenic than the conventional, chemically induced sarcomas and hepatomas in the rat. So in our system, the difference is clear-cut between the immunogenicity of sarcomas induced by MCA and the mammary carcinomas arising spontaneously or induced with carcinogens.

S. D. Chaparas: BCG content is best expressed in viable units: in milligrams and micrograms, which can vary from one vaccine to another. Do you have a conversion factor? For example, how many viable units would you find in 1 mg?

Baldwin: I can't tell you. This is GLAXO vaccine, a standard preparation.

Chaparas: Therefore, it has between 4 and 9×10^6/ml?

Baldwin: The vaccines have between 20 and 80×10^6 viable organisms/mg.

Zbar: During your vaccine contralateral tumor experiment, do you ever observe tumor growing at the vaccine site?

Baldwin: No.

Zbar: Does the strain of rats that you use give, say, a positive skin reaction to purified protein derivative if you test them that way?

Baldwin: Not very effectively.

J. E. Sokal: This bears on Dr. Alexander's question: We have explored this in a mammary carcinoma developed by Dr. U. Kim in our pathology department at Roswell Park Memorial Institute, and the answer is just as Dr. Alexander would have predicted. With this technique of immunizaiton, we have been unable to touch the tumor that metastasizes spontaneously.

G. B. Mackaness: What species was it?

Sokal: Inbred rats.

B. G. Leventhal: Did you get into any trouble giving BCG intravenously? Did any of the mice or rats die?

Baldwin: No, we didn't. I should also add that I have some data on BCG membrane preparation that Dr. Zbar very kindly let us have. When this preparation was injected intravenously, it was as effective as live viable organisms in suppresing pulmonary tumor growth.

J. W. Kreider: What are the conequences of an intravenous injection of BCG? Are extensive granulomatous lesions produced in the lungs? What are the long-term effects? Are there any emphysematous changes?

Baldwin: I can't really answer that. What I can say is that the suppression of the growth isn't due only to granulomatous response because then one would expect to get suppression of both the antigenic and nonantigenic tumors, but in the long term I think this could be a problem.

Taylor: How much time does it take in controls for the histologic appearance of microscopic nodules after the intravenous injection of tumor cells, and how does that relate to the timing of the intravenous injection of BCG?

Baldwin: I'm not sure. If we inject tumor cells alone, the animals will die about 25 or 30 days later.

Taylor: I don't mean dying. I mean the actual morphologic evidence of the tumor cells proliferating in the lungs. How far along can you prevent their progression?

Baldwin: We've only done this so far to about day 10 after the lodgment of the tumor cells. At day 10, we can still suppress growth of tumors at the lung.

Unidentified speaker: May I answer a question that was not answered, i.e., the effect of intravenous BCG in the lung?

Now, normally in guinea pigs, small granulomas form very quickly after BCG is injected; they disappear in a short time even with 10 mg of BCG. On the other hand, if you give the cell wall oil preparation intravenously, you get tremendous granulomas which practically fill the lung and these last a long time.

G. B. Mackaness: In the rat the granulomas would be even more evanescent.

A. Goldin: Many of these transplantable tumors are quite subject to caloric restriction in terms of their

ability to either take or grow. Is there any evidence of toxicity with BCG that might result in caloric restriction, which might account for at least some of these results?

Baldwin: I didn't have time to go through the data. But in fact, if you use this sort of immunotherapy in a model where you inject tumor cells on one side of the animal and tumor cells with BCG on the other side, you eventually get a tumor-specific component to the suppression. Thus, for example, animals so treated and rejecting both the challenge inoculum and the BCG-mixed inoculum will then become specifically immune so they will not reject a challenge of another sarcoma immunologically different. So there is a control in all these tests to show that the response seen is a tumor-specific immune response.

Terry: This is a comment rather than a question. And, Dr. Baldwin, it's not addressed to you because you are well aware of it: In terms of our thinking during the day, we must distinguish between immunoprophylaxis and immunotherapy.

Now, Dr. Zbar was talking about immunotherapy. That was an established tumor model. Part of Dr. Baldwin's data is based on immunoprophylaxis and part is based on immunotherapy. As we progress, we should not confuse these two concepts because both present quite distinctly different problems.

R. B. Herberman: Do you have any information about the changes in in vitro parameters of cell-mediated immunity in these animals?

Baldwin: No. We are now looking at the cell-mediated responses up to the time that the BCG is given so we can say what BCG is doing in terms of the cell-mediated immunity and serum-blocking factors at the time the BCG is given.

B. H. Waksman: This is also a question about mechanism. If you are having difficulty in causing tumors to regress when you inject BCG 7–10 days after tumor challenge, possibly the animal has started to synthetize circulating antibody against tumor antigen and this antibody is producing an enhancement which destroys the BCG-effected cell-mediated immuity. You could check that very easily by simply giving antibody passively to animals which are at the same time being given BCG.

Baldwin: These tumors have also been studied by my group for their immune parameters during growth. We do know, for example, that by day 5 animals receiving an inoculum of tumor cells have both circulating sensitized lymphocytes and serum-blocking factors in their circulation. So we do know that, when we start to give the BCG, the host has recognized the cells and has mounted an immune response. Thus, if we take lymphocytes from these animals, we can adoptively transfer the immunity and also can use the lymphocytes from these animals for colony-inhibition tests with plated tumor cells.

Also, we can take serum from these animals to show that it contains blocking factors which will abrogate the lymphocyte-mediated toxicity, so at that point, when the treatment is given, the animal is in the state of having responded immunologically to that tumor.

Unidentified speaker: Do you regard your intravenous lung model as a variation of the intralesional model from the viewpoint that, if you inject tumor cells intravenously, they lodge in the lung capillaries and that the intravenously injected BCG may lodge in pretty much the same anatomic spot?

Baldwin: This was really the logic of doing this experiment.

Unidentified speaker: Do you think there is an intimate interaction between the BCG introduced intravenously and tumor cells lodged at a previous time? There is a geographic problem here, a random distribution, I imagine.

Baldwin: This needs to be looked at.

Mackaness: Are there more questions for Dr. Baldwin, even of a technical nature, at this stage?

G. L. Bartlett: Intravenous injection with BCG under certain circumstances can interfere with the development of some immune reactions. Dr. Zbar has used this technique to temporarily prevent delayed hypersensitivity to BCG. Do you find you're walking a tightrope here in terms of the dose of BCG used intravenously? Have you ever seen anything suggesting that you are overwhelming or interfering with cellular immunity?

Baldwin: We haven't looked at it in that sense. I don't think so, but the evidence is such that, when we have an antigenic tumor, and with the doses we are giving, we can get a good suppression of the tumor growth.

Protective Effects of BCG and Other Bacteria Against Neoplasia in Mice and Hamsters[1,2]

Paul Lemonde,[3] *Institute of Microbiology and Hygiene of Montreal, Laval-des-Rapides, P. Quebec, Canada*

SUMMARY—In AK mice given BCG when young, spontaneous leukemia was repressed: The incidence was decreased, and development was retarded. When BCG was administered to older mice, spontaneous leukemia was repressed, but only in males. When BCG was combined with other interfering factors, such as fasting, fighting, or pregnancy, leukemia was repressed still further. When BCG was given in association with Cytoxan to preleukemic mice, the malignant process was impeded and the incidence of leukemia deaths was considerably lowered, more so when the agents were combined than when either agent was given alone. BCG counteracted the induction of tumors by polyoma virus in hamsters and mice. In mice bearing transplanted polyoma virus-induced tumors, when BCG was combined with surgical removal of the tumors, the incidence of recurrences was reduced. The protective influence of BCG is attributed to stimulation of immune responses to neoplastic tissue. Other bacteria known to stimulate immune responses were also investigated. *Bordetella pertussis* delayed the onset of leukemia in AK mice. *B. pertussis* and *Corynebacterium anaerobium* hindered the induction of tumors by polyoma virus in hamsters: The incidence was reduced, and the survival time of tumor-bearing animals was significantly prolonged.—Natl Cancer Inst Monogr 39: 21–29, 1973.

THIS PAPER is a survey of our studies on BCG; part of these studies has been published and will be summarized or referred to; part is new and will be outlined. Our work on BCG began in 1960. Halpern, Biozzi, Old, and their associates found that BCG counteracted some transplanted tumors (*1–3*). I reflected that BCG could similarly affect spontaneous neoplasms and tumors induced by viruses, which are more natural. As spontaneous neoplasms, lymphoid leukemia of the AK mouse was investigated; as

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[2] Supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

[3] I thank Dr. A. Guérault, Dr. M. Quevillon, and Dr. G. Charette, who participated in the experiments with *Bordetella pertussis*, and Professor A.-R. Prévot, of the Pasteur Institute, Paris, who made useful suggestions and supplied the preparation used in the experiment with *Corynebacterium anaerobium*.

virus-induced tumors, those provoked by the polyoma agent were studied.

METHODS

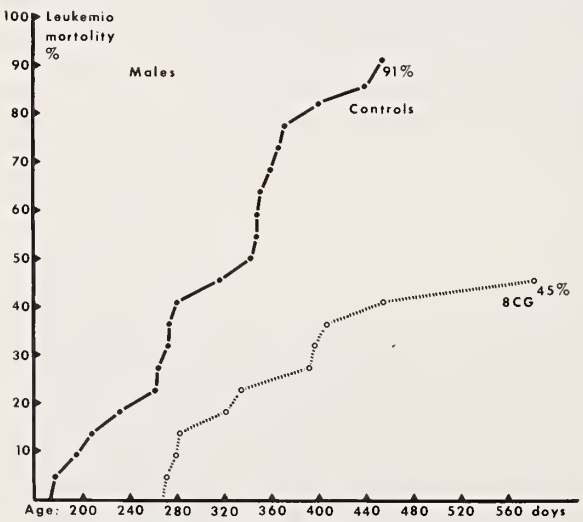
In the following experiments, living BCG was administered once, at another site from the tumors and at another time than oncogenic materials. This method differs from the methods of other investigators, who inoculated BCG into the tumors or gave it mixed with tumor cells. I used lyophilized BCG from the strain maintained in our Institute. The lots used were tested periodically; they contained an average of 6 million viable bacilli/mg wet weight. I used inbred AK and C3Hf mice, from our sublines as described earlier (*4*), and outbred Syrian hamsters, from the colony maintained in our laboratory.

EXPERIMENTS

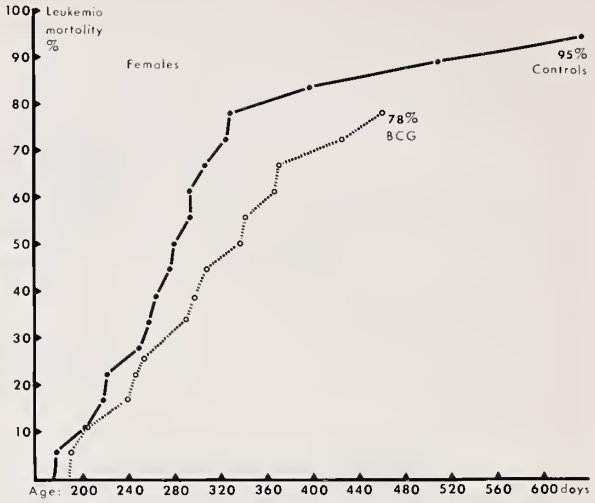
BCG and Spontaneous Mouse Leukemia

BCG Alone

AK mice received an intravenous (iv) injection of BCG, 1 mg wet weight/kg body weight, when they were 10 weeks old (71 days ± 1.3 SE). The incidence of spontaneous leukemia was decreased in these animals compared to controls. In mice that did develop leukemia, the onset of the disease and death therefrom were delayed. Text-figures 1 and 2 graph the results. In males,



TEXT-FIGURE 1.—Incidence of leukemia in male AK mice given BCG when young and in controls; 22 mice in each group. Incidence was decreased by half with BCG and death was delayed in mice that died of leukemia.



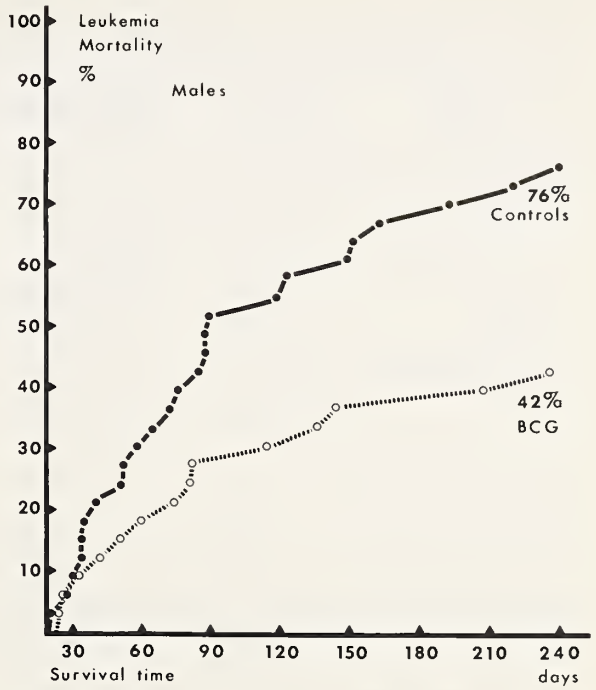
TEXT-FIGURE 2.—Incidence of leukemia in female AK mice given BCG when young and in controls; 18 animals in each group. Results were similar to those in males (text-fig. 1), but differences were not significant.

the incidence of leukemia was reduced to half, and the survival time of leukemic mice was prolonged. In females, the results were similar to those in males, but less marked. In fact, the differences in incidence and survival time between females given BCG and controls were not significant. However, 3 female controls developed leukemia very late, a rare occurrence in AK mice. Unfortunately it happened in this experiment, which reduced the difference in survival between the 2 groups. Had not those controls died so late, the prolongation of survival in females given BCG would have appeared more significant.

While we were performing these experiments, Old et al. in a comprehensive paper (*5*) reported a "modest" delay in the appearance of leukemia in BCG-infected AKR mice. We found not only a good delay in the appearance but also a decrease in the incidence of leukemia. Since our first results were published (*6*), I have administered BCG several times to AK mice, as controls for animals undergoing various treatments, and observed, as a rule, a protective effect: reduced incidence or delayed appearance, or both.

In a further experiment, BCG was inoculated, in the same manner, to older AK mice, i.e., males 275–350 days old and females 225–300 days old.

The incidence of leukemia was again decreased, almost to half in males (table 1). The mean survival time of all males that died of leukemia was not changed with BCG, but the latent period was actually prolonged, as can be seen by a comparison of the survival among the animals that received BCG and among the same number of controls or by the results as presented in text figure 3. In females, BCG had no significant effect.



TEXT-FIGURE 3.—Incidence of leukemia in male AK mice given BCG when old and in controls. With BCG, the incidence was decreased almost to half, and leukemia deaths occurred later.

BCG and Physiologic or External Factors

I administered BCG to young AK mice as in the first experiment and subjected the animals to other factors previously shown to hinder leukemia: fasting, pregnancy in females, and fighting in males. The rationale, methods, first results, and a discussion of the mechanisms involved were reported (7). Since then, other experiments were performed. Table 2 summarizes the main results. The incidence of leukemia was strikingly reduced in males; the mean survival time of test mice was not significantly different from that of controls, due to the few males that developed leukemia. In females, the incidence of leukemia was reduced, and the survival time of leukemic animals was significantly prolonged.

TABLE 1.—Effect of BCG on spontaneous leukemia in older mice

Animals—age when given BCG	Incidence of leukemia (%)	Survival time of leukemic mice (mean days ± SE)		
			Survival after BCG administration	
		Age at death	All mice	First 14 or 26 mice with leukemia*
Males, 275–350 days old:				
Controls	25/33 (76)	393.9±12.4	93.2±12.5	48.8±5.4
BCG	14/33 (42)	391.4±20.2	94.1±18.0	94.1±18.0
	$P < 0.005$			$P < 0.05$
Females, 225–300 days old:				
Controls	31/31 (100)	365.9±18.6	118.2±19.0	81.3±10.0
BCG	26/27 (96)	337.0±12.3	81.6±10.9	81.6±10.9
		Not significant		

* Number of males or females that died of leukemia in the BCG groups.

Further experiments are in progress to study such factors more thoroughly, with several groups of controls and in mice undergoing various physical exertions, e.g., swimming or running substituted for fighting. Thus far, these physical exercises are not as successful as fighting.

BCG and Chemotherapy

The idea of combining immunotherapy and chemotherapy was attractive. I undertook experiments of that sort on mouse leukemia and

TABLE 2.—Effect of BCG associated with other factors on spontaneous leukemia in AK mice

Groups and management	Incidence of leukemia (%)	Survival time of leukemic mice (mean days ± SE)
Males:		
Controls	32/36 (89)	290.2±12.2
BCG + fasting + fighting	4/37 (11)	285.8±34.6
	$P < 0.005$	Not significant
Females:		
Controls	38/38 (100)	266.1±14.0
BCG + fasting + pregnancy	26/35 (74)	354.5±23.2
	$P < 0.005$	$P < 0.01$

presented preliminary results (*8*). The combined treatment was applied first to preleukemic AK mice. In reality, most AK mice are in a preleukemic phase, since they carry a leukemogenic agent even before birth (*9*); they are in a state of latency and almost all of them will eventually develop leukemia. I call more particularly preleukemic the phase immediately preceding the stage of clinical leukemia. The latter is recognized by hyperplasia of superficial lymph nodes—determined by palpating inguinal, axillary, scapular, or cervical nodes (I do not use palpation of the spleen, which I think is not reliable)—hunched posture, bulging breast (enlarged thymus), shortness of breath, ruffled hair (mice no longer groom themselves), and of course lymphocytosis (not always, for there are instances of aleukemic leukemia). The preleukemic phase can be assessed by a decrease in body weight and in the number of lymphocytes in the blood, changes of amino acids in the plasma, etc.

Methods.—Into 4 groups were divided 135 mice, males > 275 days old and females > 225 days old (table 3). BCG was given iv, 1 mg wet weight/mouse, on the 1st day. Cytoxan was administered intraperitoneally (ip) 7 days later, at 100 mg/kg of body weight/animal and then weekly at 50 mg/kg.

Results.—The incidence of leukemia deaths was lower in mice given BCG than in controls, still lower in animals treated with Cytoxan, and lowest in mice receiving both agents. The mean survival times of mice that died of leukemia did not differ significantly from one group to another, but the development of leukemia was progressively delayed, as can be seen in the last column of table 3 or by the results given in text-figure 4.

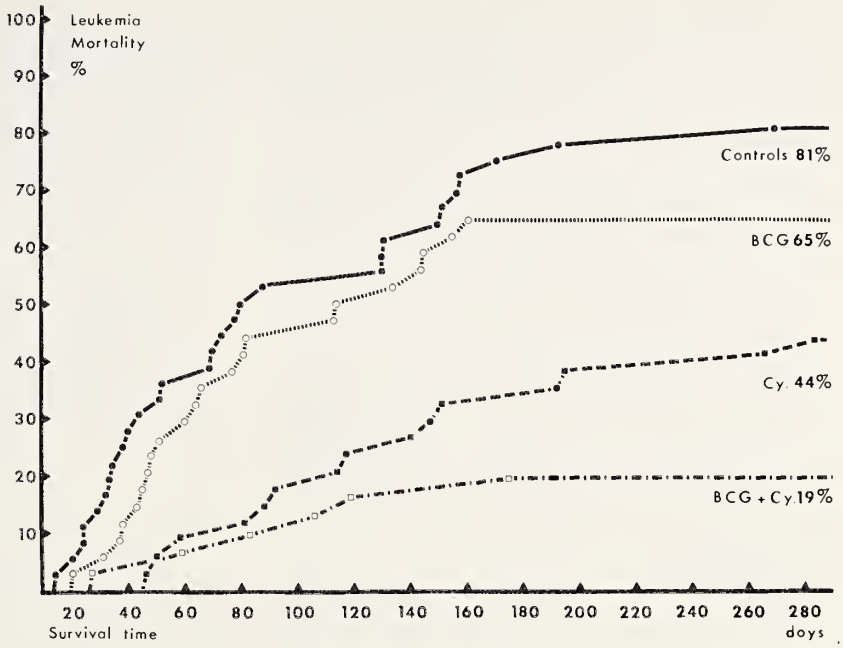
Discussion.—Various questions may be raised concerning these results. 1) If the mice were in a preleukemic phase, why did the controls not all die of leukemia? *Answer:* The animals were not actually all preleukemic. We did not go to the trouble of performing tests to see whether the mice were definitely in the preleukemic phase. We just took mice that were at an age when several animals of the same line have already developed leukemia, but that were not clinically ill, as assessed by palpation of lymph nodes and by the other signs mentioned previously. We assumed that a large proportion of mice were preleukemic. In fact, they were 81% so—the percentage of controls that died of leukemia. This figure is the point of reference to which other percentages are compared.

2) In AK mice in general, the incidence of leukemia is about 90%. In our subline, when

TABLE 3.—Effect of BCG and Cytoxan in preleukemic AK mice

Groups and management	Leukemia deaths (%)	Survival time of leukemic mice (mean days ± SE)	
		Age at death	Survival time after beginning of experiment of first 6 mice with leukemia* (significance over group 1)
1. Controls	29/36 (81)	382.3±16.6	23.8±2.6
2. BCG	22/34 (65)	367.5±14.1	36.5±3.1 ($P < 0.02$)
3. Cytoxan	15/34 (44)	402.4±18.9	69.2±8.3 ($P < 0.001$)
4. BCG + Cytoxan	6/31 (19)	351.2±19.6	94.8±20.9 ($P < 0.01$)
		Differences not significant	

* Number of mice developing leukemia in the fourth group.



TEXT-FIGURE 4.—Incidence of leukemia and survival time of leukemic mice after beginning of experiment, in AK mice treated with BCG, or Cytoxan, or both. Progressive decrease of incidence and delayed occurrence of death from leukemia were seen with the different treatments.

last calculated, it was 91%. Why is it only 81% in this experiment? *Answer:* A number of mice die of leukemia before the age at which they were taken for the trial. By using older mice, we select a group wherein the proportion of animals that will become leukemic is lower. Should we choose mice 500 days old, the proportion of leukemic animals would be $\leq 10\%$.

3) What happened to mice that escaped leukemia? *Answer:* They succumbed to diseases of old age: nephritis, amyloidosis, heart disorders, etc. Among the animals receiving Cytoxan, some may have died of intoxication by the drug. Symptoms were seen such as alopecia, cystitis, etc. This cause of death might be deduced also from a comparison of the survival time in these mice and in those that did not receive Cytoxan but also escaped leukemia (table 4): The survival tended to be shorter with Cytoxan. However, this factor could not be important, because the difference is obviously not significant.

Further to the experiment just described, I have tried various other schedules of treatment. I am not prepared to report on them at present, but it is interesting to note that in one of them, arranged on the same pattern as the first one, a certain enhancement of leukemia occurred, instead of a reduction: a slight increase in incidence and a significant acceleration in development. The only difference was that BCG was administered iv in the former trial and half ip and half subcutaneously (sc) in this one.

TABLE 4.—Survival time of AK mice that escaped leukemia with or without Cytoxan

Groups	Number of mice	Survival time after beginning of treatment (mean days ± SE)
Controls	19	162.1±16.1
Cytoxan	44	138.0±13.2 Difference not significant

I also tested the effect of combined immunotherapy and chemotherapy on mice in the leukemic stage. The results so far are ambiguous. With the combined treatment, the incidence of leukemia deaths was decreased, and the survival time was slightly but significantly prolonged. However, the mice in which leukemia regressed died just the same, at about the same time as those dying of leukemia. Apparently, they did not succumb to intoxication by the drug, because the same doses did not harm healthy mice of the same strain. Perhaps they died of intoxication by the products of rapid disintegration of leukemic tumors or of the sudden elimination of too much lymphoid tissue.

BCG and Tumors Induced by Polyoma Virus (4)

Hamsters received an ip injection of BCG, 0.4 mg wet weight/animal, when 7 days old. Polyoma virus was inoculated ip 2 weeks later. Table 5 summarizes the results. The incidence of tumors was considerably reduced in hamsters given BCG, and the survival time was prolonged in the animals that did develop tumors.

TABLE 5.—BCG and tumors induced by polyoma virus in hamsters

Groups and management	Animals with tumors (%)	Survival time of animals with tumors (mean days ± SE)
Polyoma virus	27/31 (87)	134.6±24.9
BCG + polyoma virus	15/42 (36)	190.0±20.4
	$P < 0.005$	$0.08 < P < 0.09$

C3Hf *mice* received an ip inoculation of polyoma virus within 24 hours after birth and, 4 days later, an ip injection of BCG, 0.4 mg wet weight/animal. Table 6 summarizes the results. The incidence of tumors was not changed by BCG infection, but the survival time of tumor-bearing mice was significantly prolonged. That the incidence was not reduced is probably because BCG was given after the virus. Since mice are susceptible to tumor induction by polyoma virus only a short time after birth, we first inoculated the virus into newborn animals and then administered BCG.

TABLE 6.—BCG and tumors induced by polyoma virus in mice

Groups and management	Mice with tumors (%)	Survival time of mice with tumors (mean days ± SE)
Polyoma virus	20/22 (91)	188.7±17.6
BCG + polyoma virus	24/27 (89)	240.6±19.1
	Not significant	$P < 0.052$

BCG and Surgery

C3Hf mice received a sc implantation of 3 million living syngeneic polyoma tumor cells from a line maintained in tissue culture. All developed tumors. A third of the mice were used as controls (table 7). In another third, I injected BCG iv, 1 mg wet weight/animal, 2 weeks after implantation. Then 14–20 days later, the tumors were removed surgically in animals of the last 2 groups. The incidence of recurrences was 60% in untreated animals and 47% in those given BCG. If we consider males and females separately, BCG prevented recurrences only in the males, in which the incidence dropped from 67% to 20%.

TABLE 7.—Effect of BCG and surgery on tumors (transplanted polyoma tumors in C3Hf mice

Groups and management	Incidence of tumors (group 1) or recurrences (groups 2 and 3)		
	All mice (%)	Males (%)	Females (%)
1. Implanted tumor cells	20/20 (100)	9/9 (100)	11/11 (100)
2. Implanted tumor cells + surgical removal	12/20 (60)	6/9 (67)	6/11 (55)
3. Implanted tumor cells + BCG + surgical removal	9/19 (47)	2/10 (20)	7/9 (78)

Mechanism of BCG Effects

The investigators who found antineoplastic effects of BCG had undertaken their work on the basis that BCG stimulates immune processes, and they attributed their results to such a stimulation. The amounts of various humoral antibodies to oncogenic viruses were increased in BCG-treated animals (*1*). But the effects on neoplasms are due to a stimulation of cell-mediated immunity toward neoplastic tissue as such, i.e., toward specific tumor antigens. We reported experimental evidence supporting that view (*10*), and I will not now discuss this point further.

However, it may be interesting to call attention to a recent event. In a first experiment with hamsters, described in (*10*), BCG stimulated immunity to transplanted polyomatous tumors in animals immunized with polyoma virus. In a repeat experiment, hamsters were immunized with irradiated cells. In this case, instead of a protection, we got an enhancement of tumor growth, and when BCG was given before immunizing cells, the enhancement was increased: Tumors developed earlier and faster. Thus, in certain instances, BCG might promote tumor development, possibly still by stimulating immunity, but humoral immunity, i.e., enhancing or blocking antibodies.

Bordetella pertussis and Spontaneous Mouse Leukemia

In addition to our work with BCG, we have experimented with other bacteria known to stimulate immune processes (*11, 12*). One of these is *B. pertussis* (*13*). AK mice, 38–43 days old, received an injection of live pertussis bacilli, by the intracerebral (ic) route, at the LD50, which had been established for these mice in preliminary trials as 80 U/animal. In a second group, animals were given killed bacilli, as pertussis vaccine, 10^{10} organisms ip/mouse. Controls received the suspending medium of live bacilli ic, or the medium of killed bacilli ip, or no inoculation. Table 8 shows the results.

Of 48 mice that received the LD50 of live bacilli, 27 died within a few days and the remainder served as test animals. The incidence of leukemia was not appreciably changed in any group. But in animals given live pertussis organisms, the leukemia appearance was delayed and the survival time was significantly prolonged. Killed bacilli had no effect.

B. pertussis and Polyoma Virus-Induced Tumors

In Newborn Hamsters

Methods.—Hamsters were managed as shown in table 9. We administered ic or ip live pertussis bacilli to each animal at 3 days of age at a dose of 10^5 U. We gave 3×10^8 killed bacteria ip. Polyoma virus was inoculated ip into all hamsters when 7 days old, and killed bacilli were injected on the same day in group 2.

Results.—The development of tumors was retarded, and survival time was significantly prolonged in animals that received pertussis bacilli, either live or killed, ip before the virus. Killed bacilli given ip on the same day as the virus (group 2) or live organisms injected ic before the virus (group 3) had no effect. The incidence of tumors was not reduced by any treatment.

Discussion.—That pertussis had no effect when given on the same day as the virus, or when inoculated ic, may be explained by the possibility that the bacilli had not enough time to disseminate and to influence immune defenses before the virus induced lasting neoplastic transformation. Likewise, how is it that pertussis exerted a protective effect only in relation to survival time, and not on the incidence of tumors, whereas the same bacilli markedly reduced the incidence of malignant changes in older hamsters, as we shall see in the next experiment? Perhaps the animals were too young for their immune system

TABLE 8.—Effect of *B. pertussis* on spontaneous leukemia in AK mice

Groups and inocula	Incidence of leukemia (%)	Survival time of leukemic mice: Mean days ± SE	Survival time of leukemic mice: Significance of difference over group 1
1. Live bacilli (LD50)	19/21* (91)	344.8±26.1	—
2. Killed bacilli (vaccine)	30/30 (100)	271.3±10.5	$P < 0.02$
3. Controls: Medium of live bacilli	28/30 (93)	284.6±13.4	$P < 0.05$
4. Controls: Medium of killed bacilli	27/30 (90)	271.1±13.0	$P < 0.02$
5. Controls: No inoculation	29/30 (97)	286.8±11.0	$P < 0.05$

*48 mice were given LD50; 21 survived and served as test animals.

TABLE 9.—*B. pertussis* and polyoma virus-induced tumors in newborn hamsters

Groups and management	Incidence of tumors (%)	Survival time of animals with tumors after virus inoculation (mean days ± SE)
1. Polyoma virus: Controls	12/15 (80)	30.6±1.9
2. Killed bacilli ip + virus on same day	16/16 (100)	35.0±2.6 (NS)*
3. Live bacilli ic + virus 4 days later	28/28 (100)	32.9±8.6 (NS)*
4. Live bacilli ip + virus 4 days later	28/29 (97)	52.0±6.5 ($P < 0.01$)*
5. Killed bacilli ip + virus 4 days later	16/16 (100)	51.8±10.2 ($P < 0.051$)*

*Statistical significance of difference over group 1. NS = not significant.

to respond more effectively, or the interval between administration of stimulating bacteria and inoculation of polyoma virus was too short for the immune processes to develop soon enough before the progress of neoplastic growth.

In Older Hamsters

Since killed pertussis bacilli had the same effect as live organisms in the previous experiment, we used only killed bacteria in the present trial. Bacteria were given ip to 14-day-old hamsters in the form of pertussis vaccine, 3×10^9 bacterial U/animal. Controls received the suspending medium. We inoculated polyoma virus ip into all animals 14 days later. In group 3, we again administered bacilli to the animals on the same day as the virus (5×10^9 U) and gave a third injection (same dose) 14 days later (table 10).

The incidence of tumors was well decreased in hamsters given pertussis bacilli. In the animals developing tumors, these tumors appeared later and grew more slowly; accordingly, the survival time was significantly prolonged. Giving the bacteria three times instead of once did not improve the results. The bacilli seem to act before the virus; once tumors are established, not much can be done.

A few investigators have found that *B. pertussis* inhibits neoplasia [references in (*10*)]. However, other workers have found that this microorganism might enhance tumor growth, e.g., Hirano et al. (*14*) and Floersheim (*15*).

Corynebacterium anaerobium and Polyoma Virus-Induced Tumors in Hamsters

C. anaerobium is a species of anaerobic corynebacteria similar to *C. parvum,* which was used in various studies [references in (*10*)]. Like other anaerobic corynebacteria, it stimulates the reticuloendothelial system, and it was found to be the most active in this respect (*16*). The preparation used was made and supplied by Professor Prévot, from the Pasteur Institute in Paris. It is a suspension containing 2 mg dry weight of heat-killed bacteria/ml in saline with 0.2% formalin.

Hamsters, 30 or 31 days old, received the following 3 ip injections of the suspension, at 3-day intervals: 0.25, 0.3, and 0.4 ml, respectively. Controls received the same volume of saline-formalin. On the same day as the last injection, I inoculated polyoma virus ip into all animals.

Table 11 shows the results. In hamsters given *C. anaerobium,* the incidence of polyoma changes was decreased by a factor of 3, and the

TABLE 10.—*B. pertussis* and polyoma virus-induced tumors in older hamsters

Groups and management	Incidence of tumors	Survival time of animals with tumors after virus inoculation (mean days ± SE)
1. Controls: Polyoma virus	21/25 = 84%	66.0±10.2
2. Killed bacilli once + virus	12/25 = 48% ($P < 0.05$)*	159.7±21.7 ($P < 0.001$)*
3. Killed bacilli three times + virus	14/25 = 56% ($P < 0.05$)*	136.7±21.0 ($P < 0.01$)*

*Statistical significance of difference over group 1.

TABLE 11.—Effect of *C. anaerobium* on neoplastic changes induced by polyoma virus in hamsters

Groups and management	Incidence of neoplasia (%)	Survival time of animals with neoplasia after virus inoculation (mean days ± SE)
Controls: Virus only	20/25 (80)	206.3±27.3
C. anaerobium + virus	7/25 (28)	353.4±59.2
	$P < 0.005$	$P < 0.05$

survival time of animals developing neoplasia was significantly prolonged. In addition, metastases were observed in 4 controls but in none of the test animals.

CONCLUSION

BCG and other immunity-stimulating bacteria exert interesting inhibitory effects on spontaneous leukemia in mice and on malignant changes induced by polyoma virus in hamsters and mice. A few instances of enhancement of neoplasia have been seen. We do not understand all the conditions under which these agents induce either protection or enhancement. More experimental work has to be carried out to clarify this problem.

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DISCUSSION

G. B. Mackaness: Thank you, Dr. Lemonde, for detailing your experience with another animal model. Are there any questions for Dr. Lemonde?

B. G. Leventhal: Could you detail your successful Cytoxan-BCG schedule? How did you give BCG in relation to the Cytoxan?

P. Lemonde: In the experiment I reported, BCG was given 7 days before Cytoxan. The first time 100 mg/kg Cytoxan/body weight was given, and then each week 50 mg/kg body weight was given.

D. W. Weiss: One should be precise in using the term "enhancement," and should distinguish between "enhancement" in classic immunologic terms, i.e., the presence of certain antibody factors in serum which protect

tumor cells from cytotoxic immune elements and accelerated or enhanced tumor growth for nonimmunologic reasons. One can conceive of mechanisms other than an immunologic one for growth enhancement, and in the rest of our discussion we might bear in mind this distinction.

I also want to re-emphasize the importance of dosage with regard to the resistance and enhancement balance. Years ago we reported from our laboratories similar effects with living BCG and mycobacterial fractions reducing or retarding the onset of spontaneous mammary tumors in inbred mice. The dosage of the agents was crucial. Up to a certain level, protection was marked; beyond this level, enhancement was marked. This apparently was immunologic enhancement, though I am not entirely certain of this. So, dosage of nonspecific stimulators in relation to resistance-enhancement equilibria may be another key factor to be considered in continuing discussions on this topic.

Lemonde: And route, too.

Weiss: Certainly that, too. I alluded to these points because they related to your presentation.

M. A. Chirigos: How old were the AK mice that you used in the combined Cytoxan-BCG studies?

Lemonde: They were in the preleukemic phase, i.e., the males >300 days old and the females >225 days old.

F. M. Berger: You have shown that tumors can be affected by these 3 different agents: those derived from corynebacteria, those from BCG, and those from pertussis. Is there any evidence that the same substance in all these agents is functional? Or is there any evidence that the same immunologic mechanisms are being stimulated by these various bacterial extracts?

Lemonde: I am not now prepared to answer these questions.

Berger: Is there any indication that a combination of 2 of these agents would be better than any one of them alone?

Lemonde: In 1 of the 7 schedules I mentioned, I gave BCG; 2 months later *Corynebacterium;* and 1 month later pertussis. This schedule was no better than the schedule with BCG alone.

G. L. Bartlett: This is in response to the last question: We have used a model system in guinea pigs which detects tumor-specific immunity by an effect of a vaccine on the growth of a simultaneous challenge of living tumor cells at a distant site. A vaccine of BCG mixed with tumor cells produces a very strong, dramatic tumor-rejection immunity. A vaccine of tumor cells mixed with killed *Bordetella pertussis* does not affect the growth of the challenge. Adding *B. pertussis* to the BCG-tumor cell vaccine abrogates the immunogenicity of that vaccine.

S. D. Chaparas: Male mice fight if you put them in a cage, while female mice don't usually. When mice fight, they bite each other. They have all sorts of lesions all over them and are probably predisposed to intercurrent infections. Possibly could these intercurrent infections have given your animals this added immunity that you observed in your experiment?

Lemonde: No, because when they fought too much we isolated them. There was no infection.

Chaparas: But there were many lesions on them?

Lemonde: No.

Chaparas: Bloody marks?

Lemonde: Few. We isolated them before.

Mackaness: To answer Dr. Chaparas' question: Your experiments will have to be done with completely isolated animals. That is the only way you'll get an answer.

Lemonde: I did this with males but not with females. Males were isolated in all these experiments except the ones in which fighting was the factor studied.

Weiss: With regard to Dr. Berger's question, one important point must be made here. Many of us working in the area of so-called nonspecific stimulation of tumor resistance have been working on the operative hypothesis that one is really dealing with a nonspecific stimulation of immunologic activity which is then manifested by heightened resistance against tumors. But if one wishes to play the devil's advocate—and I don't particularly like to do so, but I think one should—some suggestive evidence is now accruing—I refer especially to the work of P. Minden and some others—that there are indeed very broad cross-reactivities antigenically between a variety of mycobacterial antigens and the antigens of many other microorganisms. It appears from some recent work reported by Field, in a workshop in London 2 weeks ago on tumor immunology, that much cross-reactivity of some microbial antigens may even encompass a variety of mammalian cells. Just conceivably then what we are referring to as nonspecific protection may be at least in part not at all nonspecific but dependent on broad antigenic cross-reactivity.

R. W. Baldwin: I'd just like to return to Dr. Bartlett's point. We have recently been using one of our sarcoma systems to screen those adjuvants, and the data at the moment are a little confusing. If we mix tumor cells with BCG, we get suppression of tumor growth, which promotes a tumor-specific immune response in that the animals are specifically immune to that tumor but will not reject challenge of another chemically induced sarcoma. If we do the same experiment using *Corynebacterium parvum,* the mixed inoculum is rejected, but the animals subsequent to being given an injection are not immune to the challenge with the same tumor.

The same also occurs if we use double-stranded RNA preparations to suppress tumor growth. So there is a difference in the way in which BCG functions and the way in which some of the other adjuvants function.

A. Goldin: I worry a little about the lack of dose-response data in various experiments reported today. For example, with the AK mice, in the controls only about 80% of the animals get leukemia. Now the data would be considerably more convincing if one could demonstrate a dose-response relationship with respect to the BCG, one with respect to the Cytoxan, and then one with respect to the combination.

Lemonde: I agree.

P. Salk: Can you give more details about the schedules

of inoculation of BCG that produced tumor suppression as opposed to enhancement?

Lemonde: I cannot give more details than what I gave in my talk. I am now studying this problem.

E. Rosenberg: Dr. Lemonde, how do you explain the relative ineffectiveness of BCG in females as opposed to males, and has this difference been found in any of the other models?

Lemonde: It is known that leukemia in AK mice appears earlier and more often in females than in males. This difference has been attributed to the fact that estrogens are proleukemic in mice and androgens are antileukemic.

J. U. Gutterman: I'd like to follow-up on your answer to Dr. Leventhal's question about Cytoxan and BCG. You said you give BCG before the Cytoxan. Does that mean it does not work after Cytoxan? Or did you do the experiment only one way?

Lemonde: I did the experiment the other way too: giving Cytoxan first and then BCG.

Gutterman: When was the BCG given after the Cytoxan?

Lemonde: Four days.

Leventhal: Did it seem to work that way also?

Lemonde: Since the experiment is still in progress, I cannot give you the final outcome.

Gutterman: Studies by G. A. Currie and K. D. Bagshawe demonstrated that *C. parvum* given 12 days after Cytoxan resulted in a 70% cure of a mouse fibrosarcoma. Timing was critical, and either earlier or later administration of the adjuvant was less effective.

Lemonde: That's right. That is why I have tried—and I am continuing to try—various schedules of treatment.

F. C. Sparks: We have decreased the tumor incidence or prevented tumor growth in mice by immunizing them with BCG before tumor injection. We have also successfully treated tumors with intratumor injection of BCG.

However, we have had enhancement in several different models. Since we did not have a metastasizing tumor model available, we injected into the left leg a dose of 3-methylcholanthrene-induced fibrosarcoma cells that would grow in 90% of mice and into the right leg, one-half that dose. We wanted to see if injecting BCG into the tumor site in the left leg would exert a systemic effect on the smaller tumor inoculum in the right leg. Chicago Research BCG, 0.1 ml, was used and injected before detection of palpable tumor.

Mice with large tumors received the BCG. The tumors in these mice were significantly larger than those in the control mice which did not receive BCG. The tumors in these BCG-treated mice became detectable 2 weeks earlier and killed the mice 2½ weeks earlier than those in the control mice. Because tumors grew faster on both sides, this was not granuloma formation due to intratumor injection of BCG.

W. D. Terry: To enlarge on that and to see if other people have similar experience, when you say you gave half of the dose intraperitoneally and half subcutaneously as opposed to all intravenously, then in that model you were getting some enhancement, however it is defined.

Now we also have experience with mice in which intraperitoneal BCG under certain circumstances gave enhancement, i.e., tumor coming up sooner although the final endpoint was the same. Is this a general phenomenon in the rat and in other species? Is intraperitoneal administration more prone to give enhancement than other routes of administration?

Sparks: This experiment was the only one in which tumor growth was increased this dramatically. We've had 3 other experiments in which tumor was noted to grow faster after immunotherapy. However, in most of our experiments, immunotherapy has either suppressed tumor growth or had no effect.

Mackaness: You don't know what conditions applied—

Sparks: The tumors used were moderately or weakly immunogenic. BCG was injected into these tumors either before or after they become palpable.

J. W. Kreider: We have been studying the effects of Phipps strain BCG (Trudeau) on the B16 melanoma, and we have had similar results. In most cases, intralesional injection has little or no effect. But in some experiments with the use of BCG as adjuvant for a tumor cell vaccine, we have observed significant enhancement of growth.

M. Chase: Just what percentage of the animals had the enhancement?

Sparks: All of the animals that got it. There were about 10.

J. W. Thomas: Did you try another BCG in either of the enhancement experiments just reported?

M. G. Hanna, Jr.: Using viable Phipps strain of Trudeau BCG, we have attempted immunotherapy of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced skin carcinomas in hamsters. The injections were intralesional, and we never got regression of tumors; indeed, sometimes we got enhanced tumor growth.

It must be remembered that the intralesional BCG injections were done when the hamsters definitely had suppressed cell-mediated immunity as a consequence of the DMBA treatment. Histopathologically, when we autopsied those animals given intradermal injections of BCG, we found very widespread granulomatous disease, and the animals were classified as having miliary tuberculosis. This widespread BCG infection was not found in normal animals given intradermal injections of viable BCG; however, the survival and growth of BCG in normal hamsters were quite prolonged in contrast to the short life of viable organisms in guinea pigs. Similar results in rats and mice verify the fact that viable BCG survives for months and grows well in normal mice and rats in comparison to the short life of the organism in guinea pigs.

The point was made earlier in the discussion that the immunotherapeutic effect of BCG is a function of the immunologic status of the animal. Whether the BCG can cause a systemic disease or whether it can be a mediator of protection in a therapy or prophylaxis model depends on the abilities of the animals to effectively handle the BCG. This is particularly true in

animals immunologically suppressed as a result of a tumor or carcinogenic treatment.

It is difficult to visualize focusing immunity on or in a tumor when BCG is growing rapidly throughout the animal and the immune system is being diluted or compromised by the growth of the organism. Therefore, in many rodent systems, one will achieve an antigenic competition with this microorganism which would be detrimental to a tumor therapy model, which may cause enhancement of tumor growth as a result of divergence of the immune system.

Chaparas: There's one factor we haven't considered. We have been thinking of blocking antibody, etc., but we must also consider the possibility of antigenic competition. The normal animal may have a certain amount of immunity with which to cope. If the BCG competes with this, then this might permit the tumor cells to multiply at a rate faster than in animals not given BCG.

Mackaness: This is a critical point. There has been very little said so far about the dosage.

Unidentified speaker: The same sort of situation can be seen with inert or killed mycobacteria which already take away the possibility of the infectious parameter.

As a matter of fact, it is a tricky situation, and you can shift from one position to another. But this can even be observed with the same type of tumor, with the same type of mice, and with different dosage levels of killed BCG or even of better defined preparations, etc. This problem raises both theoretical and practical points, and one should try to evaluate it.

Mackaness: Essentially what is being said here is that circumstances exist in which the host may be busier getting rid of BCG than it possibly should be.

I'd like to mention an anecdote in relation to this. One can adoptively immunize animals against an infectious disease; but if you raise an exudate in the peritoneal cavity by injecting casein or something of the sort into the peritoneal cavity, the animal dies from the infection. So this is analogous.

C. M. Pinsky: Maybe we can know the doses in some of these cases. Dr. Sparks, did you say 1 cc?

Sparks: 0.1 ml. These mice in other experiments had a positive response to about half of that or 10 times that, so that's not a lethal dose.

Weiss: Concerning this question of antigenic preemption, during some studies on the ability of the methanol-extraction residue fraction of BCG to counteract induced states of immunodepression, we observed a rather consistent pattern. If one first exposes mice to sublethal irradiation and then attempts to correct the immune depression induced by some of these questions, the effect commonly seen is *reduced* immunologic recuperation of the animals. If the situation is reversed and pretreatment is effected before irradiation, one finds a positive protective effect.

Various data suggest that we may be dealing here with some kind of antigenic competition, especially in animals in which immunologic responsiveness has already been reduced by the insult of radiation or chemotherapeutic treatment.

Immunotherapeutic Studies in Mice With the Methanol-Extraction Residue (MER) Fraction of BCG: Solid Tumors[1,2]

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SUMMARY—Investigations were conducted on the therapeutic effects of the methanol-extraction residue (MER) fraction of inactivated BCG organisms against solid tumors in 2 inbred strains of mice. MER was used alone and combined with X-irradiation and chemotherapy; the parameters and schedules of treatment were varied widely. Tested were 2 long-transplanted, carcinogen-induced fibrosarcomas, a mammary tumor virus (MTV)-negative mammary carcinoma, and an MTV-positive mammary carcinoma of recent origin. Efficacy of treatment was assayed in 2 test systems: established neoplastic isografts and newly initiated, simulated local-recurrence tumors. MER given alone effectively retarded tumor development and prolonged host survival in only a few instances; it only rarely accelerated tumor growth or shortened survival. In contrast, MER combined with irradiation or chemotherapy significantly inhibited tumor growth rate, significantly extended the life of the hosts in a large proportion of the experimental groups, and had no negative effects. Such combination therapy was often more effective than treatment with X-rays or chemotherapy alone and proved to be efficacious even under conditions in which the single therapy was inactive. The experimental conditions most favorable to the demonstration of therapeutic activity by MER alone and in combination schedules were similar to the circumstances under which MER has been shown to elevate immunologic reactivity, and especially cellular immune responsiveness, to defined and seemingly unrelated antigens. The present observations are thus compatible with the assumption that MER stimulates the immunologic apparatus nonspecifically and thereby promotes immunologic attack against antigenic neoplastic cells. It also appeared that MER benefits the host by protecting against toxic side effects of other therapy on hematopoietic tissue. The implications of these observations for immunotherapy of human cancer are discussed.—Natl Cancer Inst Monogr 39: 33–54, 1973.

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DURING THE past several years, extensive studies have been in progress in our laboratories on the biologic properties of a methanol-extraction residue (MER) of phenol-killed BCG organisms. This mycobacterial fraction can modulate profoundly, quantitatively and qualitatively, the immune response to defined antigens, administered separately in time and place, and can significantly elevate the resistance of experimental animals to microbial pathogens and to the development of neoplastic isografts and of spontaneously occurring tumors. The parameters of the protective activities of MER are very similar to the parameters of its stimulations of immunologic responsiveness, especially of IgM and cellular reactivities. In many model systems, the effects elicited by MER are greater and last longer than those induced by living or inactivated intact BCG organisms; there are indications that the mechanisms by which the fraction affects immunologic capacity and states of resistance differ in at least some respects from those brought into action by the undisrupted parent bacilli. These findings have been summarized in 2 review articles on MER (*1, 2*); newer experimental observations are described in other communications (*3–5*).

Thus far, most of the experiments on the intervention by MER in host-tumor relationships have been of a prophylactic nature. Only lately has more detailed experience accrued on the effects of MER against already established neoplastic cells. Thus it was found that the material can, depending on the circumstances of administration, either raise or lower resistance of C57BL/6 mice against leukemic cells induced by the radiation leukemia virus (*4*); very early trials with MER in patients with acute myelocytic leukemia suggest some therapeutic activity (*6*). The present communication reports the first results obtained from a large series of new investigations on the therapeutic activities of MER alone and MER combined with radiation therapy and chemotherapy against mammary carcinomas and fibrosarcomas in inbred mice.

We constructed a number of different model systems and widely varied the parameters of treatment and challenge in the hope of approximating in at least some test systems certain aspects of the host-tumor relationship of spontaneously occurring neoplasia in man. No single animal model can be expected to mirror accurately the human situation (*7*), but by broadening the experimental test circumstances and reducing as far as possible blatant artificialities, we hope to achieve a degree of relevance.

Throughout these studies, the therapeutic efficacy of MER was tested against tumor challenges very rapidly fatal in the control animals. This was done to obtain a picture of MER efficacy under the most stringent conditions and, thereby, to avoid any unrealistic impression of the potential usefulness of the material. Although only limited effects could therefore be anticipated, and were obtained, they provide grounds for hope that more impressive results may be expected when MER is used in subjects confronted with a less rapidly lethal tumor challenge.

MATERIALS AND METHODS

Animals

Male and female mice of 2 inbred strains were used, BALB/c and C3HeB. The BALB/c animals, which are free of the known mammary tumor viruses—mammary tumor virus (MTV) and nodule-inducing virus (NIV)—were from the breeding colony of the Hadassah Medical School, where the line was started in 1968 from breeding pairs generously supplied by Dr. K. B. DeOme of the Cancer Research Genetics Laboratory of the University of California in Berkeley. The C3HeB animals were obtained from the breeding colony of the Weizmann Institute, Rehovoth, Israel. The animals were introduced into the experiments at 6–10 weeks of age.

Diet consisted of standard mouse pellets and water given ad libitum.

The isogenicity of the animals in each strain was confirmed repeatedly throughout these studies by the successful takes of second-set skin grafts exchanged between randomly chosen pairs of mice of the same sex.

Tumors

The tumors were of isogenic origin, maintained by repeated passage in isogenic hosts, with the exception of the MTV-positive mammary carcinoma used in the third transplant generation. The following tumors were studied: 1) a benzo[*a*]pyrene (BP)-induced fibrosarcoma in C3HeB males; 2) 3-methylcholanthrene-(MCA)-induced fibrosarcoma in BALB/c females; 3) a mammary adenocarcinoma, which originated in an outgrowth of a hyperplastic alveolar nodule appearing in a hormonally hyperstimulated BALB/c female and which was thus free of MTV and NIV [immunization with this tumor,

designated D7T4S, tends to lead to enhancement rather than to resistance (*8*)]; and 4) a mammary adenocarcinoma which developed spontaneously in a multiparous BALB/cfC3H female and which was thus infected with the mammary tumor viruses.

In most studies with the mammary carcinomas, we used tumor D7T4S. We based this choice on 2 considerations: 1) Since progressively growing neoplasms in man might also represent populations of neoplastic variants selected during the disease for the property of favoring the evocation of enhancement-promoting rather than resistance-promoting immune reactions, we wished to examine the ability of MER to reverse this tendency; and 2) mammary carcinomas of mice carrying the MTV show abundant RNA viral particles which seem to be unrelated to the numbers of any such oncogenic viral agents detected in some malignant cells of man. However, to obtain a comparative impression of efficacy of treatment with a recently arisen, virus-infected neoplasm, parallel experiments were also conducted with a BALB/cfC3H mammary carcinoma in BALB/c hosts; previous observations showed that MTV-infected tumors express potent antigenic properties related to the presence of the virus (*9–11*).

All the tumors used had been shown in other, direct immunization studies to possess some degree of tumor-associated antigenicity. As is well known, however, neoplasms maintained by repeated passage are likely to lose tumor-associated antigenic properties, and they may well undergo selection for a variety of "virulence" characteristics. We decided to use such tumors in most of these initial studies so as to mimic the clinical situation in which the population of malignant cells to be eradicated by direct therapy or by a bolstering of host defenses frequently represents very distant descendants of the clone (s) which underwent the original transformation.

In further studies, to be reported later, tumors were used in the first 3 transplant generations, so as to reproduce the rarer clinical circumstances of treatment of recent, or very slowly growing, cancers.

Tumors were measured several times weekly with calipers, and tumor volumes calculated from the formula $V = 0.4ab^2$, where a is the major and b the minor diameter bisecting the palpable growths at right angles to each other (*12*).

In several exploratory experiments, subgroups of mice were killed periodically, and their tumors weighed. The data on tumor growth so obtained were very similar to those derived from the calculations of tumor volumes, and it thus appears justified to rely on calculated tumor volumes as a reasonable approximation of tumor development.

MER

Several lots of MER were used, some prepared in our laboratories and some by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey. The preparation of the material has been detailed elsewhere (*1, 13*). All lots used showed high activity in the standard *Klebsiella* protection assay (*14*) and in several immunologic stimulation (vis-à-vis known antigens) models. The MER was stored at 4° C over a desiccant until shortly before use. Aqueous suspensions were prepared by the dispersion of the dry material in pyrogen-free isotonic saline by means of a high-speed VirTis stirrer; the suspensions either were used immediately or were maintained frozen at −20° C until use.

In many experiments, MER was injected subcutaneously (sc), at one or more sites at each time of administration. It has been found previously that the intraperitoneal (ip) route of MER introduction is more effective in some (though not in all) test systems; as this route is not the most feasible in man, however, the sc one was chosen for many of the animal studies. For comparison, other routes of administration were also used in a number of instances, and several groups received the fraction both directly into the tumor and elsewhere.

Therapeutic Irradiation

Therapeutic irradiation was by means of X-rays; a Picker Vanguard instrument was used, 125 kV, HVT 0.6 mm Al, FSD 19 cm, with a calculated dose at the tumor site of 2000 or 3000 R in 4–6 minutes. Before irradiation, animals were lightly anesthetized with ether. In the isograft experiments, the animals were not given body shielding but in the simulated local-recurrence studies, lead shielding was used so that whole-body exposure to irradiation was reduced to a minimum; nonetheless, a certain amount of exposure of body areas other than the tumor focus could not be ruled out.

In further experiments to be reported later, the dosage of radiation was varied.

Chemotherapy

The following chemotherapeutic agents were used:

Cyclophosphamide	(Taro Pharmaceutical Industries, Haifa, Israel)	I mg/mouse
5-Fluorouracil (5-FU)	(Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.)	0.25 mg/mouse
Vinblastine sulfate (VNB-S)	(Eli Lilly & Co., Indianapolis, Ind.)	0.006 mg/mouse
Methotrexate (MTX)	(Lederle Laboratories Division, American Cyanamid Co.)	0.01 mg/mouse
Thio TEPA	(Lederle Laboratories Division, American Cyanamid Co.)	0.025 mg/mouse

In all the experiments here described, chemotherapy was administered once or twice. In later experiments, to be published subsequently, different amounts of these agents and repeated treatment were given.

Experimental Design

Two types of experiments were conducted. 1) The effects of MER, therapeutic irradiation, and chemotherapy alone and in combination were tested against established tumor isografts. 2) The treatments were directed against "simulated local-recurrence tumors." Saline-treated or untreated control groups were included in each experiment.

Tumor isograft model.—Fragments of the tumors (1 mm^3) were implanted sc in the left inguinal region of each animal. When the tumors in approximately half the animals in each experiment reached a volume of 50–120 mm^3, treatment was begun. MER, X-irradiation, or chemotherapy was administered alone once or twice or in combination. Combined treatment was always given sequentially, by different schedules, for up to 4 applications but with no group receiving the same treatment more than twice. Treatment lasted between 10 and 25 days after tumor implantation. The total amount of MER administered ranged from 0.2 to 1.6 mg at each treatment. The growth rates of the tumors and survival times of the animals were followed by inspection of the mice and tumor measurement every 3 or 4 days.

Simulated local-recurrence model.—Tumor fragments were implanted as described above. When tumor volumes reached approximately 50 mm^3 in half the animals of each experiment, *all* the tumors were removed surgically, as described previously (*15*). At the time of removal, a 1 mm^3 fragment of each animal's tumor was reintroduced into that animal, at the same site. Different schedules of treatment were used, ranging in time from 1 week before tumor removal to several weeks after. The animals were observed as indicated above.

Both the isograft and simulated local-recurrence experiments were usually terminated 40–90 days after reimplantation or recurrence, when most animals were dead or had very large tumors.

Statistical Analysis

In the simulated local-recurrence experiments, the mice were distributed randomly in the different treatment groups after tumor removal and reimplantation. In the tumor isograft experiments, each treatment group was assigned equal numbers of animals with tumors of different sizes, so that the range of tumor size was nearly equal in each treatment and control group at the beginning of therapy. Each group contained between 10 and 20 animals.

Analysis of differences in tumor volumes between the experimental and control groups and between different experimental groups in each experiment was by the nonparametric Mann-Whitney U Test. Comparisons, made at different times after treatment, were based on the absolute values of tumor volume and on periodic increments of growth. In the calculation of mean tumor volumes, all animals alive in a group at that time of reading were included in the calculation, whether or not they displayed a palpable growth; animals without palpable tumors were assigned a value of 0.4 mm^3. (Only a very small proportion of the mice died without evidence of palpable tumors, or with small tumors.)

Analysis of differences in survival time between the various groups in each experiment was by the χ^2 test. Occasional animals dying without palpable tumors were excluded from the calculations. The proportion of animals surviving was compared at different times after tumor challenge.

Differences between groups were considered significant when P was ≤ 0.05; comparisons are referred to as significant only when this criterion was met at least at one point in time.

RESULTS

Tumor Isografts

The initial series of experiments was conducted with the tumor isograft model; most of these experiments are now complete.

Tumors developed progressively in almost all the animals. In most instances growth was rapid, the mice succumbing with large tumor masses. No significant differences were observed between experimental and control groups in the rare incidence of complete inhibition of tumor growth. However, there were significant, though usually modest, differences in the growth rates of the tumors in the differently treated groups, and in the survival times of the hosts.

Treatment With MER Alone, Irradiation Alone, and MER and Irradiation

The results of several experiments with the 2 fibrosarcomas and the MTV-negative mammary carcinoma are presented in tables 1 to 5.

In table 1, the effect of treatment on the rate of tumor development is shown. MER given alone once or twice, in quantities totaling 0.2–3.0 mg (18 groups), was ineffective in all but one case, regardless of dose and route of administration, and in only one instance accelerated tumor growth; this group was given MER both ip and directly into the tumor mass. Irradiation alone and combined treatment with MER and

TABLE 1.—Effect of treatment with MER, X-irradiation, and both on development of neoplastic isografts

Treatment	Number of groups treated	Number of groups showing*		
		No significant effect	Significant retardation of tumor growth	Significant acceleration of tumor growth
BP-Induced Fibrosarcoma in C3HeB Males†				
MER alone	9	8	1	0
Irradiation alone	7	3	4	0
MER and irradiation	17	8	8	1‡
	33	19	13	1
Mammary Carcinoma in BALB/c Females				
MER alone	3	3	0	0
MER and irradiation	6	1	5	0
	9	4	5	0
MCA-Induced Fibrosarcoma in BALB/c Females§				
MER alone	6	5	0	1
Irradiation alone	2	0	2	0
MER and irradiation	6	1	5	0
	14	6	7	1

* Each experimental group is compared with the corresponding control group.

† Summary of 3 experiments.

‡ The degree of accelerated tumor growth, although significant, was slight; despite this limited acceleration, the animals in this group had a prolonged survival time compared to that of the controls.

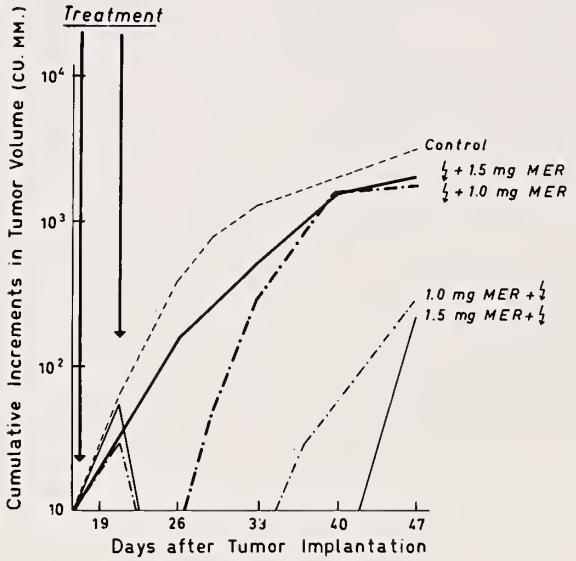
§ In this experiment, the animals receiving irradiation alone were accidentally lost.

irradiation slowed tumor development in an appreciable proportion of the experimental groups (9 receiving irradiation only and 29 receiving combined therapy). Irradiation alone never accelerated growth, and only once did combined therapy slightly, but significantly, accelerate growth; in this case, however, the survival time of the animals was somewhat prolonged.

When combined treatment was administered, no consistent differences were observed between groups given different amounts of MER (0.4–1.5 mg) once or between groups receiving the fraction sc or ip; however, 2 injections of MER combined with irradiation failed to slow tumor growth whereas a single injection was effective. MER given before irradiation was as frequently and as markedly effective as the reverse schedule in the fibrosarcoma in C3HeB animals, but administering of MER before irradiation seemed more effective in the MTV-negative mammary carcinoma (text-fig. 1).

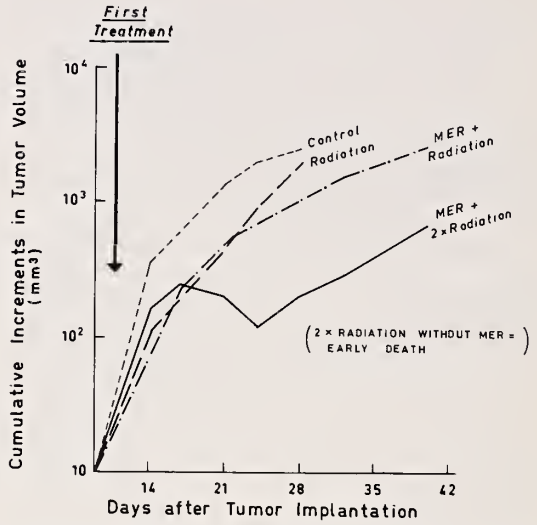
Groups given radiation therapy alone and exhibiting significant tumor retardation were compared with groups in the same experiment which had received combined treatment of significant efficacy. Only groups exposed to irradiation at the same times after tumor implantation were compared because the time radiation was administered was found to affect the results on at least some occasions (early treatment appearing to be more effective). In 8 such comparisons, there was no significant difference between combined therapy and X-ray alone; in 3, irradiation alone was significantly better; and in 3, combined treatment yielded significantly superior results. In some experiments, moreover, a large proportion of the mice given radiation therapy twice died shortly after treatment, whereas most of the mice so exposed but also given MER survived the entire experiment (text-fig. 2). Also, animals receiving MER in addition to repeated irradiation consistently appeared healthier than those subjected to repeated irradiation alone. Thus, although these findings do not permit generalization as to a greater efficacy of combined therapy with X-rays and

GROWTH OF IMPLANTS OF A MTV(-) MAMMARY CARCINOMA (D7T4S) IN BALB/c FEMALES UNDER DIFFERENT TREATMENT



TEXT-FIGURE 1.—Treatment administered on days 17 and 21 after tumor implantation. *Crooked arrows* = irradiation (2000 R). *Curves* represent the means of the cumulative increments in calculated tumor volumes for each group as a function of time after tumor implantation. All 4 groups differed signficantly from the controls. The 2 groups receiving MER first (ip) and then irradiation differed significantly from the groups receiving the reverse schedule.

GROWTH OF IMPLANTS OF A BENZPYRENE-INDUCED FIBROSARCOMA IN C3H MALES UNDER DIFFERENT TREATMENT



TEXT-FIGURE 2.—Treatment administered on days 10, 14, and 17 after tumor implantation. *Curves* represent the means of the cumulative increments in calculated tumor volumes for each group as a function of time after tumor implantation. The group receiving irradiation alone once (2000 R) (day 10) differed slightly but significantly from the controls, as did the groups given MER (1.5 mg, distributed in 3 sc sites, on day 10) and irradiation either once (day 14) or twice (days 14 and 17). Most of the animals given irradiation twice and no MER died shortly after treatment, whereas most of those irradiated twice and treated with MER survived the observation period.

MER than of X-rays alone, adding MER to radiation therapy improved the physical condition of the mice and at least in a number of experiments elevated therapeutic efficacy.

A further comparison was made in each experiment of all "equivalent" experimental groups receiving different types of treatment with each other, regardless of whether they varied significantly from their respective controls. (Where a single type of therapy was administered once or twice to both of the groups to be compared, equivalent groups are defined as those in which the different treatments were given an equal number of times and at the same intervals. Where one of the groups compared received combined treatment and the other treatment with only one agent, comparison was restricted to pairs in which the common treatment was applied the same number of times and at the same intervals. In groups receiving MER alone versus groups given combined treatment, comparison was made only between pairs given the same amounts of MER.) It appeared desirable to make such an intergroup comparison in addition to that of significantly different experimental groups with the controls, because it could be questioned whether animals untreated or given saline really represent adequate control for the administration of a bacterial moiety or irradiation; it might be argued then that a clearer, or at least a different, picture of treatment efficacy would be derived from a comparison of differently treated groups with each other. Table 2 shows all such comparisons in which significant differences were found between the experimental groups.

TABLE 2.—Comparison between equivalent experimental groups with regard to tumor growth retardation

Modality of treatment compared with other treatments (col. 1)	Number of times the treatment (col. 1) differed significantly or did not differ significantly from treatment with	
	Irradiation	Irradiation and MER
BP-Induced Fibrosarcoma in C3HeB Males		
MER (9)*—More effective than†	4	3
Less effective than†	4	8
Not significantly different from	3	8
Irradiation (7)—More effective than		2
Less effective than		4
Not significantly different from		11
Mammary Carcinoma in BALB/c Females		
MER (3)—More effective than	—‡	1
Less effective than	—	5
Not significantly different from	—	0
MCA-Induced Fibrosarcoma in BALB/c Females		
MER (6)—More effective than	—	0
Less effective than	—	4
Not significantly different from	—	2
Irradiation (2)—More effective than		3
Less effective than		0
Not significantly different from		2

* Number of groups.
† More effective and less effective always indicate significant differences.
‡ Absence of equivalent groups.

Table 2 shows that MER alone was more effective than irradiation alone as frequently as irradiation was more effective than MER (4/11 comparisons). Combined therapy was much more often superior to MER alone than it was inferior (17/21 vs. 4/31 comparisons, $P < 0.01$). Combined therapy was better than irradiation alone against the C3HeB fibrosarcoma in 4 of 17 comparisons and less beneficial in 2 of 17, whereas X-rays alone were more active than the combination against the BALB/c fibrosarcoma in 3 of 5 comparisons, the reverse not occurring in any of the 5 ($P < 0.05$).

There was also the consistent impression that single treatment with MER was more effective than double administration with the same quantities given at each time and that the smaller amounts of the fraction (0.4, 0.5 mg) were no less effective by single administration than the larger ones (1.0, 1.5 mg).

The effect of treatment with MER and X-rays on the survival of C3HeB and BALB/c mice carrying tumor isografts is shown in table 3. MER alone did not prolong significantly the survival of the BALB/c animals carrying mammary carcinoma D7T4S, but the combination treatment was effective in 2 of 6 instances; one group given only MER showed a significantly but only slightly decreased survival time. In contrast, MER alone and irradiation alone lengthened significantly, and in some instances considerably, the survival of the C3HeB males implanted with a fibrosarcoma (2/9 and 3/7 groups, respectively), and combined treatment exerted such an effect in most cases. The incidence of survival protection bestowed by the combined treatment was significantly greater than that afforded by treatment with MER only (12/17 vs. 2/9, $P < 0.05$) and greater (but not significantly so) than irradiation only (12/17 vs. 3/7). (Survival data were not available on the experiment in BALB/c females with fibrosarcoma isografts because subgroups of these animals were killed at intervals to obtain tumor weight values.)

Comparison of the equivalent groups with each other in each experiment in which survival time prolongation differed significantly from

TABLE 3.—Effect of treatment with MER, X-irradiation, and both on the survival times of mice bearing neoplastic isografts

Treatment	Number of groups treated	Number of groups showing*		
		No significant effect	Significant increase in survival time	Significant decrease in survival time
BP-Induced Fibrosarcoma in C3HeB Males†				
MER alone	9	7	2	0
Irradiation alone	7	4	3	0
MER and irradiation	17	5	12	0
	33	16	17	0
Mammary Carcinoma in BALB/c Females				
MER alone	3	2	0	1
MER and irradiation	6	4	2	0
	9	6	2	1

* Each experimental group is compared with the corresponding control group.
† Summary of 3 experiments.

that of the corresponding controls (table 4) indicated that the extent of efficacy of combined treatment was greater than that of MER alone and, in some instances, of irradiation alone. Although in other instances irradiation was as effective as the combination, administration of X-rays alone was never more efficacious.

The results of a further comparison of all equivalent groups with each other in each experiment, regardless of whether each group and its controls differed significantly in survival time, are presented in table 5. The greater protective efficacy of combined therapy is clearly evident from these comparisons. In 13 of 23 comparisons, combination treatment was better than MER alone, and in 4 of 14 it was better than X-rays alone. MER by itself was superior to combination therapy in 2 of the 23 comparisons and to irradiation alone in 1 of 8. X-ray treatment was more effective than MER in 4 of 8 comparisons and more effective than the combination in only 1 of 14. In the remaining comparisons, no significant differences in efficacy were detected. It thus appeared that, whereas MER alone was usually less effective than the other treatments, at least occasionally it yielded the best results.

It could be asked whether the frequently greater efficacy of combination therapy could be

TABLE 4.—Comparison between equivalent experimental groups, each one of which differed significantly from the corresponding controls with regard to survival times*

Treatment (col. 1)	Number of times treatment (col. 1) differed significantly or did not differ significantly from treatment with	
	Irradiation	Irradiation and MER
BP-Induced Fibrosarcoma in C3HeB Males		
MER (2)†—More effective than‡	0	0
Less effective than‡	1	2
Not significantly different from	0	0
Irradiation (3)—More effective than		0
Less effective than		3
Not significantly different from		4

* There were no significantly different equivalent groups in the experiment with mammary carcinoma D7T4S in BALB/c mice.
† Number of groups.
‡ More effective and less effective always indicate significant differences.

TABLE 5.—Comparison between equivalent experimental groups with regard to survival-time prolongation

Modality of treatment to be compared with other treatment (col. 1)	Number of times the treatment (col. 1) differed significantly or did not differ significantly from treatment with	
	Irradiation	Irradiation and MER
BP-Induced Fibrosarcoma in C3HeB Males		
MER (9)*—More effective than†	1	2
Less effective than†	4	10
Not significantly different from	3	5
Irradiation (7)—More effective than		1
Less effective than		4
Not significantly different from		9
Mammary Carcinoma in BALB/c Females		
MER (3)—More effective than		0
Less effective than		3
Not significantly different from		3

* Number of groups.
† More effective and less effective always indicate significant differences.

due to the fact that animals treated by some of the combination schedules received a larger *number* of treatments, rather than to the fact that different *types* of therapy were used. The answer is clearly negative. It has already been noted that 2 administrations of MER were usually less effective than 1 in the case of single-type therapy, and double exposure to X-rays alone at times affected survival adversely; thus increasing the number of treatments administered did not in itself constitute an advantage. Moreover, additional comparison of groups receiving combined therapy with groups given single-type therapy administered with the same frequency showed that mixed therapy was almost always more effective; this was true even when single-type treatment consisted of 2 exposures to irradiation and combined therapy of 1 treatment with X-rays and 1 with MER.

This impression was supported by a further comparison made between groups given combined therapy 3 times, and groups given combined therapy twice. Three administrations were less effective than 2 in 5 of 8 instances and more effective in 2. In 4 of the 5 comparisons in which 3 administrations proved inferior, the repeated treatment was with MER and in only 1 with X-rays; thus repeated injection of MER may be less desirable than a 1-time application even in combined treatment schedules. In the 2 cases where 3 administrations of therapy were better than 2, the repeated treatment was with X-rays. Thus therapeutic irradiation given twice to animals also receiving MER (protectively?) may represent the most favorable modality. Comparisons of combination-therapy groups given X-rays twice and MER once with groups given MER twice and X-rays once also suggested superiority of the double-irradiation, 1-time MER schedule.

These analyses thus indicate that it is not the number of times treatment is given which determines the outcome, but rather the fact of combined versus single-type therapy and the nature of the combined therapy schedule.

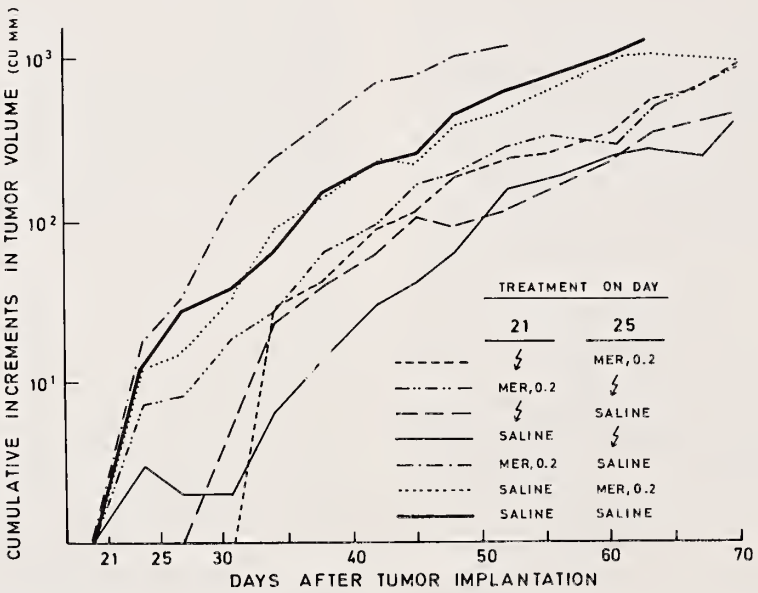
In the experiment with the MCA-induced fibrosarcoma in BALB/c females, MER was given to some groups ip and to others both ip and directly into the tumor mass. Comparison of the groups receiving MER by these means gave no clear indication that administration into the neoplastic growth offered an advantage, regardless of whether therapy was with MER alone or with MER and X-rays; in the one case of accelerated tumor growth in a MER-treated group, the fraction had been injected directly into the tumor as well as ip. In light of the recent reports by Rapp, Zbar, Borsos, and their colleagues, that introduction of living BCG and cell wall fractions into a tumor focus is requisite for a therapeutic effect in a guinea pig-hepatoma system (*16*), we decided to make addi-

tional attempts to compare the efficacy of MER administered into or adjacent to neoplastic tissue, and at other sites.

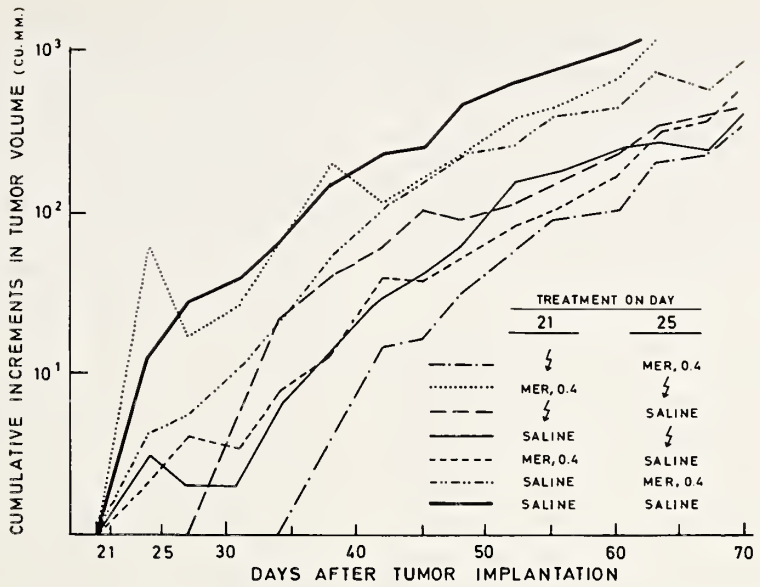
In an exploratory experiment with the BP-induced fibrosarcoma in C3HeB males, MER was administered, on the 12th day after tumor implantation, by 3 or 4 sc injections, for a total of 0.8–1.6 mg. Some animals received all the injections in the immediate vicinity of the tumor only, others at 4 sites of which one was in the tumor area, and still others at 3 sites all distal to the tumor. Control mice were given 4 sc injections of saline, one at the tumor site. Tumor development was not retarded in the animals given MER in either amount at the tumor location only. For the 2 other forms of administration, the smaller dose of MER was ineffective, whether given only at sites removed from the tumor implant or near the tumor as well as in other areas; the larger quantity did slow neoplastic growth in both instances. Thus, here again, no evidence was seen of a greater efficacy of MER administration in close proximity to the cancer.

A further experiment was then conducted on the effects of sc administration of MER distal to the neoplastic isografts, into the tumor, and by both routes simultaneously. The tumor used was a BALB/cfC3H mammary carcinoma (MTV-positive), of recent origin in BALB/c female hosts. Some of the animals were given irradiation instead of MER, and some received both forms of therapy. This tumor grew somewhat more slowly than the long-transplanted D7T4S mammary carcinoma, and a number of the animals in this experiment are still alive. It is already evident, however, that some of the modalities of treatment appreciably retarded tumor growth (text-figs. 3–5).

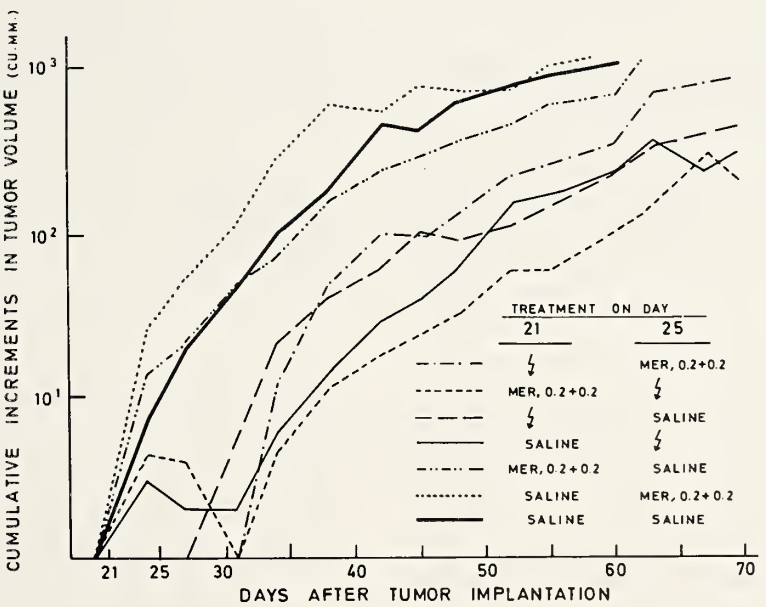
As is seen from text-figure 3, MER alone administered sc in very small amounts (0.2 mg, single injection) was not therapeutic, and in one instance accelerated somewhat the growth of the tumors. In contrast, irradiation alone delayed the initial development of the carcinoma, and combined treatment with MER and X-rays appeared to have a similar, though not superior, effect. When the amount of MER was increased



TEXT-FIGURE 3.—Growth of implants of a recently arisen MTV(+) mammary carcinoma in BALB/c females under different treatments. MER (0.2 mg) was administered by a single sc injection distal to the tumor site; irradiation therapy (*crooked arrows*) was with 3000 R X-rays. *Curves* represent the means of the cumulative increments in calculated tumor volumes for each group as a function of time after tumor implantation.



TEXT-FIGURE 4.—Growth of implants of a recently arisen MTV(+) mammary carcinoma in BALB/c females under different treatments. MER (0.4 mg) was administered by a single sc injection distal to the tumor site; irradiation therapy (*crooked arrows*) was with 3000 R X-rays. *Curves* represent the means of the cumulative increments in calculated tumor volumes for each group as a function of time after tumor implantation.



TEXT-FIGURE 5.—Growth of implants of a recently arisen MTV(+) mammary carcinoma in BALB/c females under different treatments. MER was administered simultaneously directly into the tumor and sc distal to the tumor site, 0.2 mg at each place. Saline was also injected both into the tumor and distally, sc. Irradiation therapy (*crooked arrows*) was with 3000 R X-rays. *Curves* represent the means of the cumulative increments in calculated tumor volumes for each group as a function of time after tumor implantation.

to 0.4 mg (text-fig. 4), the treatment successfully delayed tumor development, especially when it was given at the earlier interval after challenge and to an extent similar to that effected by irradiation. The combination of irradiation plus MER, with the X-ray treatment preceding, was clearly the most effective of all treatments; surprisingly, however, combination treatment by the reverse schedule was here entirely ineffective.

When MER was given in a total amount of 0.4 mg, but divided into 2 halves (one of which was injected sc distal to the tumor site and the other directly into the tumor), tumor growth was not affected (text-fig. 5). On the other hand, such administration of MER in combination treatment with X-rays was at least as effective as irradiation alone, and perhaps more so, regardless of which of the 2 treatments was applied first.

It thus appears that dosage of MER, timing of treatment, route of injection, and nature of the schedule by which additional treatment with X-rays is given are interacting parameters determining the outcome of therapy with the fraction.

Treatment With MER Alone, Chemotherapy Alone, and MER and Chemotherapy

The results of a number of experiments with the BP-induced fibrosarcoma in C3HeB males and the MTV-negative mammary carcinoma D7T4S in BALB/c females are presented in tables 6 and 7. MER was given ip, sc, and intramuscularly (im), once or twice, 1.0 mg each time; sc and im administration was distal to the tumor. Drug administration, always ip, was also given once or twice, and combination therapy consisted of 2–4 applications of different treatments. No group of animals received more than one chemotherapeutic agent.

In table 6, the effect of the different treatments on the rate of tumor development is shown. None of the treatments, of either the single or combination type, significantly accelerated tumor growth. In the experiments with the C3HeB sarcoma, MER given alone significantly retarded tumor development in 1 of 8 groups, whereas MER plus cyclophosphamide was successful in 4 of 6 groups. In contrast, cyclophosphamide alone did not exert a significant effect in 4 of 4 instances. Because preliminary study revealed the other drugs to be entirely ineffective against the sarcoma in the quantities here employed, they were tested only against the mammary carcinoma, one group of animals each being treated with a different drug alone or combined with MER. Against this mammary carcinoma, only 5-FU and thio TEPA exerted a significant effect when given alone, and the combinations of MER with either 5-FU or MTX were also effective.

The results of these experiments with regard to survival time are depicted in table 7. It is again apparent that the effects of treatment on survival time may not be identical with effects on tumor growth rate. Thus MER given alone gave significant survival protection in 1 of 8 groups but shortened survival in 2 others; in 1 of these 2 groups, however, the treatment significantly slowed tumor development. MER plus cyclophosphamide never shortened survival; in 3 of the 6 groups in which the combination was tested against the C3HeB sarcoma, and in the 1 group in which it was employed against the mammary tumor, survival protection was elicited. Cyclophosphamide alone afforded such protection to 2 of 4 groups carrying the sarcoma, but not to the group challenged with the mammary carcinoma. Against this mammary tumor, none of the chemotherapeutic agents given alone prolonged survival; indeed 5-FU, MTX, and thio TEPA shortened it. In contrast, the combinations of 5-FU–MER and thio TEPA–MER as well as cyclophosphamide–MER were significantly effective.

Combined treatment of MER with another modality of therapy thus emerges as a safe and frequently more effective means of therapeutic intervention than conventional therapy or treatment with the fraction alone. It is also seen that not only MER may on rare occasions induce an acceleration of tumor growth or a shortening of survival when given alone, but also chemotherapy can lead to such negative results when administered by itself even in barely therapeutic quantities.

Further experiments are now in progress in which mice carrying tumor isografts are being treated with larger doses of chemotherapy, alone

TABLE 6.—Effect of treatment with MER, chemotherapy, and both on the development of neoplastic isografts

Treatment	Number of groups treated	Number of groups showing*		
		No significant effect	Significant retardation of tumor growth	Significant acceleration of tumor growth
BP-Induced Fibrosarcoma in C3HeB Males†				
MER alone	8	7	1	0
Cyclophosphamide alone	4	4	0	0
Cyclophosphamide + MER	6	2	4	0
	18	13	5	0
Mammary Carcinoma in BALB/c Females				
Cyclophosphamide alone	1	1	0	0
5-FU alone	1	0	1	0
VNB-S alone	1	1	0	0
MTX alone	1	1	0	0
Thio TEPA alone	1	0	1	0
Cyclophosphamide + MER‡	1	1	0	0
5-FU + MER	1	0	1	0
VNB-S + MER	1	1	0	0
MTX + MER	1	0	1	0
Thio TEPA + MER	1	1	0	0
	10	6	4	0

* Each experimental group is compared with the corresponding control group.
† Summary of 5 experiments.
‡ Chemotherapy administered before MER.

TABLE 7.—Effect of treatment with MER, chemotherapy, and both on the survival times of mice bearing neoplastic isografts

Treatment	Number of groups treated	Number of groups showing*		
		No significant effect	Significant increase in survival time	Significant decrease in survival time
BP-Induced Fibrosarcoma in C3HeB Males†				
MER alone	8	5	1	2‡
Cyclophosphamide alone	4	2	2	0
Cyclophosphamide + MER	6	3	3	0
	18	10	6	2
Mammary Carcinoma in BALB/c Females				
Cyclophosphamide alone	1	1	0	0
5-FU alone	1	0	0	1
VNB-S alone	1	1	0	0
MTX alone	1	0	0	1
Thio TEPA alone	1	0	0	1
Cyclophosphamide + MER§	1	0	1	0
5-FU + MER	1	0	1	0
VNB-S + MER	1	1	0	0
MTX + MER	1	0	1	0
Thio TEPA + MER	1	0	0	1
	10	3	3	4

* Each experimental group is compared with the corresponding control group.
† Summary of 5 experiments.
‡ In one of these groups, the treatment significantly retarded tumor development.
§ Chemotherapy administered before MER.

and with MER. Studies have also been initiated on the effects of triple-combination treatment, with MER, irradiation, and chemotherapy. The results of one such experiment in which 5-FU was used are presented in table 8.

Table 8 shows that triple-combination treatment was significantly effective when MER and X-rays were given, in either sequence, prior to 5-FU. The degree of tumor retardation effected by the schedule, irradiation–MER–chemotherapy, was very marked. Possibly administration of 5-FU before MER or irradiation was less effective because of the systemic immunosuppressive effects exerted by the drug. Experiments are now under way to determine whether triple-combination therapy is, in fact, more effective than treatment with MER and only 1 other agent.

Simulated Local Recurrence

A much larger number of experiments was conducted employing the simulated local-recurrence system than the isograft model. Many of these studies are still in progress. A comprehensive report of the effects of single-type and combination therapy on the development and growth of recurring tumors and on the survival of the hosts in which these are simulated will be deferred until a final picture is obtained. It is already evident, however, that therapy with MER alone and with MER combined with irradiation and chemotherapy can suppress tumor development entirely in a small minority of the animals and markedly slow tumor growth and lengthen host survival in a large proportion of the remainder. Only a few experiments are presented here with the MTV-infected mammary carcinoma and with the MTV-negative tumor D7T4S in BALB/c females to illustrate the tumor retardation and host survival effects.

Text-figures 6–8 show the results of 3 consecutive preliminary experiments in which there was evident the superior efficacy of combined treatment with MER (0.4 and 0.75 mg) and X-rays over treatment by irradiation alone. (In text-figs. 7 and 8, the curves for groups receiving only MER are omitted, such treatment having had little or no therapeutic action.) Different schedules of combination therapy were effective, treatment being initiated in some groups only after removal of the primary growth and reimplantation (text-fig. 6) and in others already before surgical intervention (text-figs. 7, 8). The findings shown in text-figure 8 again indicated that smaller amounts of MER (0.4 mg) in combined treatment may be more effective than larger quantities (0.75 mg).

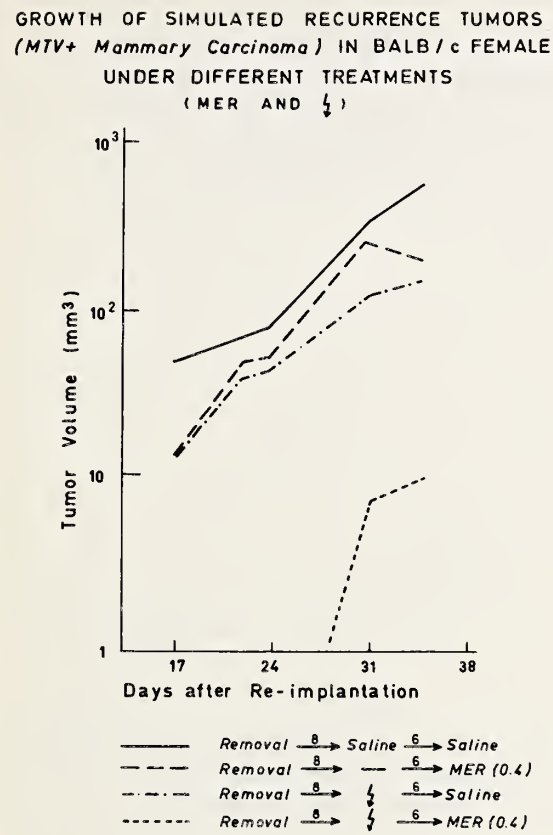
The greater efficacy of combined treatment in this system was also seen when a larger amount of MER (1.5 mg) was used (text-fig. 9). In some experiments, however, irradiation alone proved at least as beneficial as the combined therapy (text-fig. 10). Although clear superiority of X-rays given by themselves over combined treatment was seen only infrequently, the relative efficacy of the 2 modalities cannot be finally evaluated until all experiments are completed.

The effects of single-type and combined treatment on tumor growth rate and on host survival are further compared in the experiment depicted

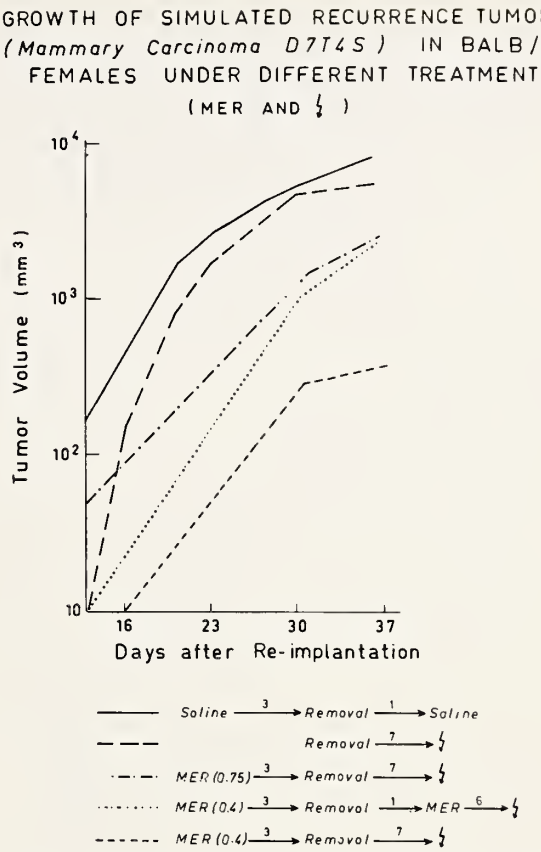
TABLE 8.—Effects of combined treatment with MER, 5-FU, and X-rays on the growth of a BP-induced fibrosarcoma in C3HeB males

Treatment* at following times (days) after tumor implantation			Effects of treatment† on	
12	15	19	Tumor growth	Host survival
0	0	0	—	—
5-FU	X-ray	MER	NS‡	NS
MER	5-FU	X-ray	NS	NS
MER	5-FU	X-ray + MER	NS	NS
MER	X-ray	5-FU	Significant retardation	NS
X-ray	MER	5-FU	Significant retardation	Significant prolongation

* Treatment: MER, im, 1.0 mg at each administration; X-rays: 2000 R; and 5-FU: ip, 0.25 mg.
† Each experimental group is compared with the control group.
‡ NS = no significant difference from control group.



TEXT-FIGURE 6.—Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *numbers over first arrows* indicate days elapsed since tumor removal and simultaneous reimplantation in situ; *numbers over second arrows* indicate days elapsed since the preceding treatment. MER was administered sc, distal to the tumor site; the amounts given are indicated by *values shown in parentheses*. Irradiation (*crooked arrow*) was with 3000 R X-rays. *Curves* represent the means of the calculated tumor volumes for each group as a function of time after tumor reimplantation. The group treated with MER and X-rays had significantly retarded tumor development.

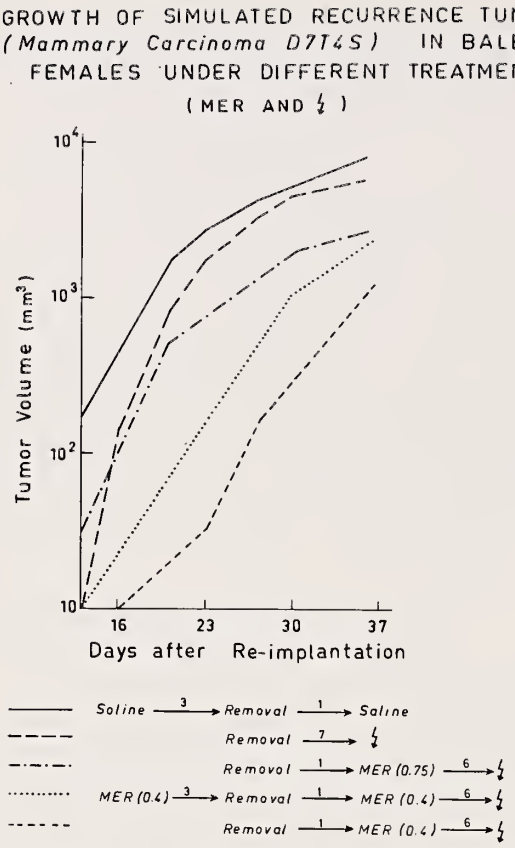


TEXT-FIGURE 7.—Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *numbers* indicate days elapsed since the preceding treatment or manipulation (tumor removal and simultaneous reimplantation). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. The 3 groups receiving treatment with both MER and X-rays had significant tumor retardation.

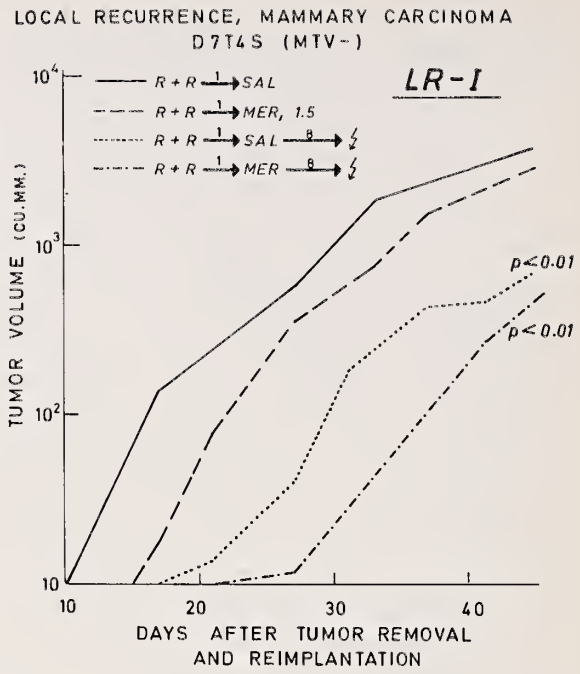
in text-figures 11 and 12. As seen in text-figure 11, several schedules of combined therapy retarded tumor development more effectively than sole treatment with X-rays; low dosage of MER and single administration again appeared to be maximally effective. In contrast, one of the groups subjected to irradiation alone showed the same prolonged survival as was bestowed by several of the schedules of combined therapy (text-fig. 12). It is thus evident once again that a given modality of therapy may have a different implication for tumor growth and host survival.

Even in experiments in which the control animals as well as most of those receiving different forms of treatment died very rapidly with large tumor masses, some schedules of combined therapy produced a definite, albeit limited, protective effect (e.g., text-fig. 13).

Reimplantation of tumor fragments immediately after removal of the primary growth represents a very unfavorable circumstance for detecting the effects of the specific immunizing experience: Many investigators have found that a period of some days to weeks must elapse between removal of an immunizing tumor and



TEXT-FIGURE 8.—Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *numbers* indicate days elapsed since the preceding treatment or manipulation (tumor removal and simultaneous reimplantation). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. The 2 groups receiving therapy by the schedules MER $\xrightarrow{3}$ Removal $\xrightarrow{1}$ MER $\xrightarrow{6}$ (with *crooked arrow* indicating irradiation) and Removal $\xrightarrow{1}$ MER (0.4) $\xrightarrow{6}$ (with *crooked arrow* indicating irradiation) had significant tumor retardation.

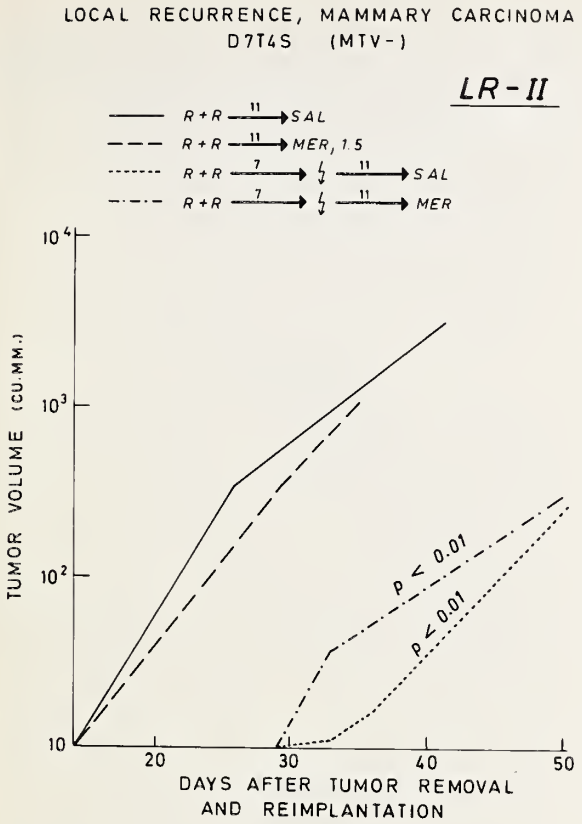


TEXT-FIGURE 9.—Growth of simulated local-recurrence tumors in BALB/c females under different treatments—MER and irradiation (*crooked arrows*). Experiment LR-I. Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *all such numbers* indicate times in relation to tumor removal and simultaneous reimplantation (R + R). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. The groups receiving combined treatment or irradiation only had significant tumor retardation.

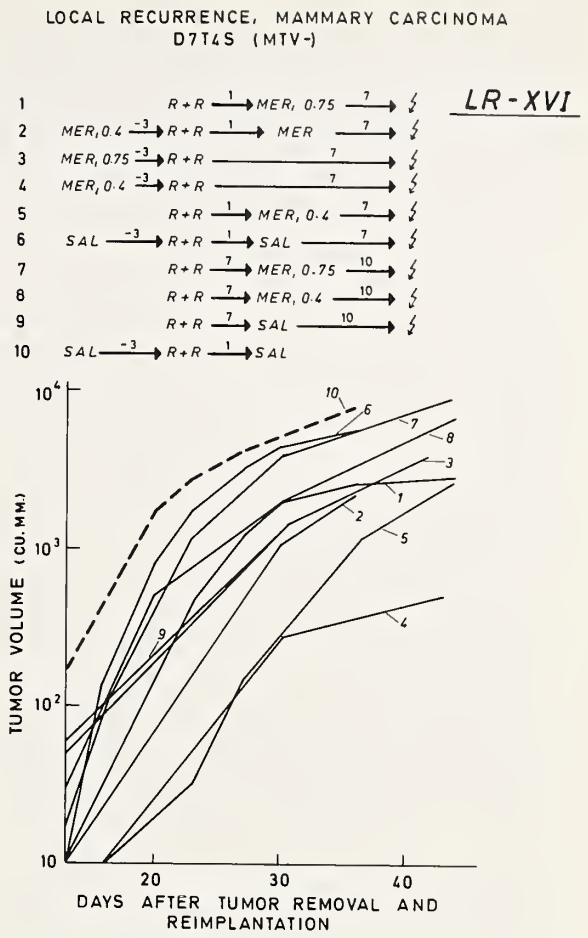
manifestation of specifically acquired resistance by the host. The present model, although it mimics the clinical circumstance of incomplete removal of a cancer and subsequent local recurrence, is thus not designed to demonstrate the efficacy of specific immunologic stimulation. Other experiments have been initiated, therefore, to analyze the efficacy of combined specific and nonspecific stimulation, with and without chemotherapy and therapeutic irradiation; in these studies, specific immunization is administered and examined under conditions more favorable to its expression.

DISCUSSION

Extensive experiments were conducted on the therapeutic efficacy against solid tumors in inbred mice of the MER fraction of inactivated BCG organisms, administered alone and in combination schedules with X-irradiation and chemotherapy. The underlying assumption of these studies was that the previously demonstrated ability of MER to heighten resistance against neoplastic cells is the outcome of a nonspecific stimulation of immunologic reactivity against tumor-associated cell-surface antigens. A large body of information on the biologic properties and behavior of the fraction strongly



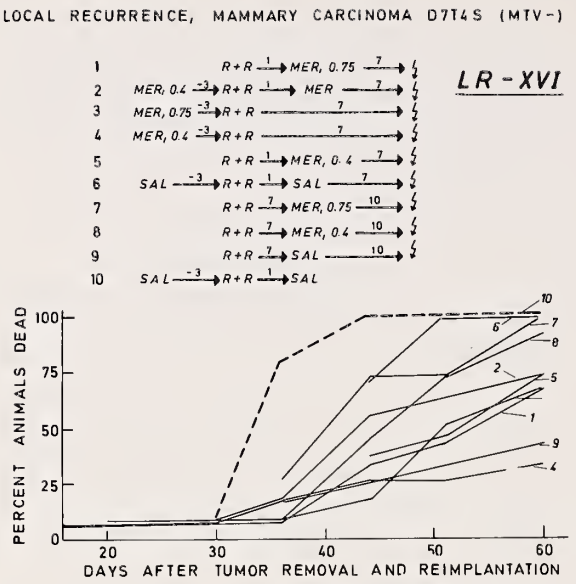
TEXT-FIGURE 10.—Growth of simulated local-recurrence tumors in BALB/c females under different treatments—MER and irradiation (*crooked arrows*). Experiment LR-II. Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *all such numbers* indicate times in relation to tumor removal and simultaneous reimplantation (R + R). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. The groups receiving combined treatment or irradiation only had significant tumor retardation.



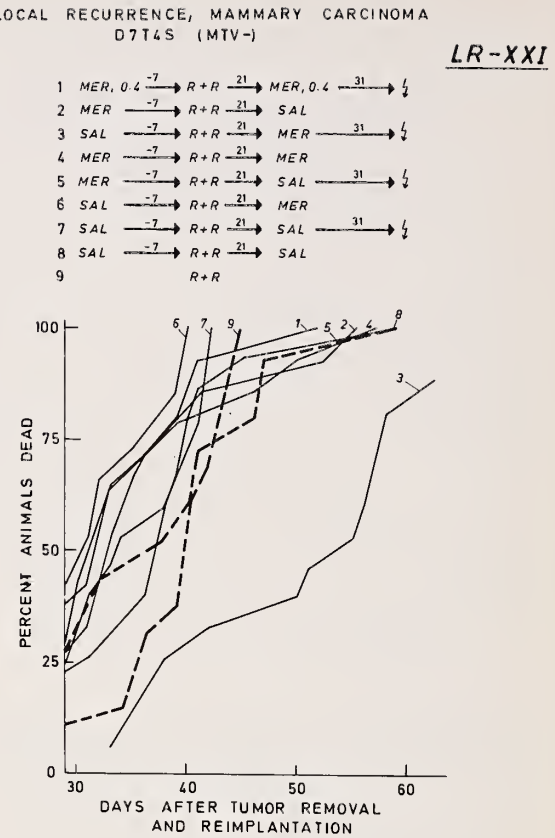
TEXT-FIGURE 11.—Growth of simulated local-recurrence tumors in BALB/c females under different treatment—MER and irradiation (*crooked arrows*). Experiment LR-XVI. Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *all such numbers* indicate times in relation to tumor removal and simultaneous reimplantation (R + R). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. Statistical analysis not performed (some animals still alive and in the experiment).

suggests that its tumor-protective capacities indeed reflect its action on the immunologic apparatus of the host (*1, 2*). The present findings show that MER has immunotherapeutic efficacy against solid tumors under the same experimental conditions which determine its stimulatory effects on immunologic responsiveness, especially of the cellular type. What remains uncertain is whether MER is truly a *nonspecific* immunologic stimulator. Recent observations by Minden et al. (*17*) point to a very broad cross-reactivity between mycobacterial antigens and antigens of taxonomically unrelated microorganisms, and such cross-reactivity may conceivably extend to mammalian cells, including transformed ones (*18*); work is now in progress in our laboratories to answer this question. If such inclusive cross-reactivity indeed exists, conceivably at least some of the supposedly nonspecific activities of MER and other tubercle bacillus entities are, in fact, specific in nature.

There are clear advantages to nonspecific immunologic intervention in cancer, or to inter-



TEXT-FIGURE 12.—Survival of BALB/c females carrying simulated local-recurrence tumors and given different treatment—MER and irradiation (*crooked arrows*). Experiment LR-XVI. The percentages of animals dead as a function of time after tumor reimplantation are shown for the different treatment groups whose tumor growth rates are depicted in text-figure 11.



TEXT-FIGURE 13.—Survival of BALB/c females carrying simulated local-recurrence tumors and given different treatment—MER and irradiation (*crooked arrows*). Experiment LR-XXI. Treatments were administered at the times (days) indicated by *numbers over arrows* in the key to the curves; *all such numbers* indicate times in relation to tumor removal and simultaneous reimplantation (R + R). Radiation was with 2000 R. *See* legend to text-figure 6 for other details. *Curves* show the percentages of animals dead as a function of time after tumor reimplantation. Statistical analysis not performed (some animals still alive in the experiment).

vention by means of very widely cross-reactive antigens over specific means of immunization. A single, broadly active agent may suffice to bestow heightened resistance to many types of antigenically alien cells, thus obviating the necessity of making a variety of tumor-cell vaccines against particular types of cancer. This might prove to be a near-impossible task if the protective antigens of human neoplasms turn out to be distinguished by a high degree of type-specific or even patient-specific individuality; antigens common to certain related animal and human tumors have been reported (*19*), including fetal antigenic determinants (*20*), but it is by no means certain that broadly effective vaccines which will not induce autoimmune pathologies can be prepared from neoplastic cells. Moreover, the fact of a progressive malignant process may in itself indicate specific or general immunologic dyscrasia, which would require more than still further exposure to the same antigens to overcome. These and other considerations pointing to the at least theoretic advantages of (seemingly) nonspecific immunologic stimulation have recently been discussed elsewhere by one of us (DWW) (*21*), and the experiences in this and other laboratories with MER have led us to invest our initial efforts at immunotherapy against solid tumors toward nonspecific stimulation. Studies have been begun on the effects of combined specific and nonspecific immunotherapy and prevention, and preliminary results have already suggested that such joint treatment may be superior to either modality alone and may prevent entirely the dangers of causing immunologic enhancement (*22*).

The findings here presented show that MER can exert significant therapeutic effects against actively growing, primary, neoplastic isografts and simulated local-recurrence tumors. MER sometimes bestowed significant resistance in terms of survival prolongation and/or retardation of tumor development even when given by itself, but combination therapy with MER and irradiation or chemotherapy was synergistically or additively effective in a much larger proportion of the groups so treated.

The tumors here used were rapidly growing, invariably progressive, and fatal in the isogenic hosts, and therapy was applied under circumstances intrinsically unfavorable for the demonstration of maximum effects. Thus, in the isograft experiments, treatment was begun when many of the tumors were already well established, and in the simulated local-recurrence model immediately after termination of the initial tumor experience, when host organisms usually are in a state of specific or even generalized immunologic unresponsiveness. Moreover, in most of the experiments here reported, the tumors were long-established cancers, repeatedly transferred by animal passage. The effects of none of the treatments were, accordingly, dramatic, but they were often appreciable and statistically significant.

A basic question that must be asked of any such experiments as are here reported is: What is the relevance of such animal models to the clinical situation in man? This question has been alluded to in greater detail by one of us (DWW) (*7*), and suffice it to state here that no single animal test system can ever represent even a near approximation of the human situation. Perhaps the most closely similar animal model would be spontaneous neoplasia in autochthonous outbred animals, but the logistic difficulties of systematic investigations with such host-tumor relationships are prohibitive. The only feasible alternative is to vary widely the systems of animal experimentation, in the hope that general principles emerging from such studies rest on a sufficiently broad base to include the human circumstance as well.

This we have attempted to do in the present experiments. The hosts were mice of 2 different inbred strains; the neoplasms included carcinomas infected with and free of a known oncogenic agent (the MTV), sarcomas induced by different chemical oncogens, and tumors both arising spontaneously and induced and transplanted repeatedly and of very recent origin; the dosage of MER, the routes of administration, and the nature and scheduling of the accompanying treatment were varied widely. Indeed, the variations of the parameters of tumor challenge and therapy were so great that even though these experiments encompassed > 150 groups of mice, totaling > 2000 animals, identical experimental conditions did not recur often. It is not possible, therefore, to derive from these findings a clear impression as to which *precise set of conditions* most consistently leads to the best therapeutic results. To obtain such a view, identical sets of test circumstances will have to be compared repeatedly, in distinct experiments, over a period of time. Such studies are under way. The need for such repetition becomes obvious when one considers that the animals used were not specific-pathogen free, and although they were ordinarily "healthy" mice, they were nonetheless subject to considerable fluctuations in the nature of their microflora. Such variations may have far-reaching consequences (*23*), especially in experiments in which the crucial test parameter is the activity of an extrinsic immunologic stimulator of microbial origin.

On the other hand, our findings do permit a number of at least tentative generalizations, which provide a basis for future experiments and clinical trials. One strong impression obtained was the frequent superiority of combined treatment. This was generally more effective than any modality of single-type therapy; therapeutic efficacy was often of a relatively higher order; activity was at times manifested even in circumstances under which MER, X-rays, and chemotherapy administered alone were wholly inactive. It sometimes appeared that MER administered before irradiation was a more desirable schedule than the reverse, but this tendency was not seen consistently. MER may be more effective when given before rather than after irradiation in the case of weakly immunogenic tumors such as mammary carcinoma D7T4S (*8*). It has been shown that MER favors cellular over humoral immune responses to weak

antigenic stimuli, but stimulates both, and perhaps antibody production preferentially, vis-à-vis strong ones (*5*). We have also observed that MER given to mice following whole-body irradiation depresses immunologic recovery as measured by responsiveness to a limited antigenic challenge, whereas MER given before irradiation has a restorative effect (*24*). Further experiments are necessary to determine whether there is commonly a greater likelihood of efficacy when MER precedes treatment with X-rays.

There was at least a suggestion from some of the simulated local-recurrence protocols that close proximity of timing between administration of MER and X-rays may be a condition for efficacy. This may not be surprising. Other experiments have shown that MER given together with tumor-associated antigens or at a time when small numbers of viable neoplastic cells are present in the host (*1, 22*) can heighten resistance, even where MER introduced prophylactically without tumor cell antigens is ineffective or leads to enhancement. Ionizing irradiation and many chemotherapeutic agents destroy target cells and presumably lead to a release of cell-surface antigens; these treatments may thus, in effect, resemble the circumstance of joint specific and nonspecific immunologic stimulation.

It was also consistently apparent that MER, even when administered without any other form of therapy, only rarely accelerated tumor development or shortened survival time, and did so when given in combination treatments in only 2 instances of all the groups here tested. In one of these, the other treatment was with X-rays; although the animals showed a slightly accelerated development of tumor growth, their mean survival was prolonged. In the other case, the combination of the thio TEPA and MER reduced survival time but did not affect tumor growth rate, and thio TEPA alone similarly shortened survival. The safety of MER treatment is thus strongly supported by the present observations, and it is of special interest that combined treatments were successful even when the challenging tumor was the MTV-negative mammary carcinoma D7T4S which in specific immunization experiments has shown a tendency to induce immunologic enhancement (*8*).

The nature of the contribution of MER to combination therapy awaits clarification. It may well be that MER and the other treatments both act against the neoplastic cells, the former by strengthening host immunologic defenses. It could also be that MER is of benefit at least in part because it protects against toxic side effects of irradiation and chemotherapy on hematopoietic tissue and immunologic function. Cotreatment with MER may thus permit greater exposure of the subject to conventional treatment without a dangerous heightening of susceptibility to microbial disease and perhaps to other pathologies arising from severely reduced immunologic capacity. It was indeed noted in at least some of our experiments that MER protected the animals against the fatal consequences of repeated exposure to irradiation. The observation that retardation of tumor growth and prolongation of host survival do not always go hand in hand is compatible with the possibility that the mode of therapeutic action of MER in combination treatment is a twofold one: stimulation of immunologic attack on tumor target cells and defense of the host against the damaging effects of irradiation and chemotherapy. Under some experimental conditions, only one or another of these effects may be expressed.

The optimum doses of MER were usually in the range of 0.4–1.0 mg, given by a single injection; larger quantities and repeated administration were often less effective. Introduction of MER directly into the tumor was not, in most instances, a requirement for efficacy. The conditions of MER effectiveness here seen are thus very similar to those which define the activity of the material in many other models of elevation of states of resistance and of immunologic function (*1, 2*). Other studies (*1, 2*) also indicate that there is no direct relationship between optimum dosage of MER and body weight of animals of the same or of different species. This is not surprising with regard to a substance which remains in the tissues for long periods after administration (*1*) and which acts indirectly by stimulating existing host defense mechanisms. There is the problem, however, of fixing the optimum dose range for human patients.

In studies recently initiated in Jerusalem (*6*)

with MER in patients with acute myelocytic leukemia, we are administering MER repeatedly, once a month, by the intradermal route, 1.0–2.0 mg at each administration. All patients receive standard therapy as well. The MER schedule is based on considerations coming from these and other animal experiments. Because small amounts of MER seem to favor cellular versus humoral immune responses vis-à-vis a limited antigenic challenge and to reduce even the remote likelihood of tumor enhancement, we chose a quantity of the material near the optimum range for small laboratory animals, and thus most probably far below the threshold of over-dosage for man. At each administration, MER is injected in 5–10 sites, to facilitate the involvement of a larger number of immunologically reactive cells and to reduce the extent of in situ inflammation. Repeated administration, even though it is often less effective than single treatment in animal models, was suggested by the usually much longer (in absolute terms) duration of malignant disease in man and by the demonstration of other investigators that repeated treatment with other tubercle bacillus entities is requisite in patients (*25*). However, the extrapolation of favorable conditions of treatment from the animal model to man is, at best, only an intelligent guess, and means must accordingly be devised to determine empirically, and perhaps in each patient, the optimum parameters of treatment. We are doing this in our clinical trial by measuring twice every month the immunologic capacity of treated and control patients; we are using a broad battery of tests for primary and secondary responsiveness of humoral and cellular type to a variety of antigens, including the autochthonous neoplastic cells.

Animal studies of the type here described are an indispensible first stage in the design of new immunotherapeutic approaches to the treatment of human malignant diseases, in that they suggest, even if only in outline form, the principles and boundaries of safe and effective therapy. It also seems clear, however, that the final formulation of parameters of treatment in man must be based on detailed studies in the human subject.

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DISCUSSION

G. B. Mackaness: Are there any questions for Dr. Weiss?

M. Chase: Have you used any of the extracted organisms in parallel experiments instead of the methanol-extraction residue (MER)?

D. W. Weiss: Yes. In many of the earlier experiments, we compared living or killed BCG, extracted organisms, and the methanol extracts. All of these substances have some activity in at least some models. The only agent we found to be highly and consistently effective, however, seems to be this MER. I don't know why this is so, but it may be because, during exhaustive methanol extraction, one may be "opening up" something in the bacterial cell wall and exposing it so that it can exert biologic activity.

S. R. Rosenthal: First, is this with oil or without oil? Secondly, what is the most effective route of administration?

Weiss: The MER in all of these studies was given without oil. We have used either a preparation suspended by us or a preparation formulated here at the National Cancer Institute in a much finer state, in an aqueous excipient.

The most effective route of administration seems to depend on the system. In some systems, intraperitoneal is more effective than subcutaneous or intravenous, but generally, at the optimum dosage, one can get near-maximal activity by any of the routes we have tried: subcutaneous, footpad, intramuscular, and intraperitoneal. However, one may have to go up in dosage to obtain the same effect by the subcutaneous route as one could obtain by the intraperitoneal route.

L. Chedid: I was wondering whether the questions raised by you gentlemen could not be resolved by the possibility that your chemical procedure is not only revealing certain activities but also eliminating others. Whole cells may provide a "package deal" committing the host in too many directions and thereby baffling his responses. So my precise question would be: Do you know what activities you are getting rid of? For instance, are they tuberculin sensitivity, sensitization to endotoxins, or granuloma formation? And just to be sure that I am on a good wavelength, am I correct in assuming that MER is the residue remaining after extraction of organisms containing both ghosts and also a compact aggregate of the cells?

Weiss: With regard to the latter point, you are entirely right. The MER *is* the residue of methanol extraction. But I understood Dr. Chase's question to refer to intact bacterial organisms, not subjected to intensive extraction and not as broken up.

With regard to your other point, MER will sensitize both guinea pigs and humans to tuberculin, but very much less so than living or whole-killed BCG. Granuloma

formation is induced at the site of administration, but it is not commonly induced in draining lymph nodes unless very large quantities are used. Sensitivity to endotoxin may be induced but apparently to a very much lesser extent than by living BCG; on this point, I am not certain.

I agree with you that we may well be removing some negative materials in the methanol-extraction, acetone-washing procedures. We find that the extracts themselves have low activity and that small, suboptimum quantities of MER, together with the extract, sometimes act synergistically.

Chedid: But what was removed could have lost its functional structure, and you could, therefore, subsequently not find it in the supernatant.

Weiss: I look upon the whole bacillary organism as a parcel of biologically active material; I'm also sure that overlap is considerable in the chemical and physical structures of the active materials in the various crude preparations that have been used. That is one of the obvious deficiencies of dealing with crude preparations. We cannot yet relate particular biologic activity to defined molecular entities with a few exceptions, notably your and E. Lederer's work on adjuvant-active, defined moiety.

Chedid: Lastly, may I ask if it's antigenic?

Weiss: We have looked for this and not found a positive response, but I don't think the question is closed. We have never found evidence of a triggering of autoimmune disease in a variety of strains of mice and guinea pigs, but I cannot rule out the possibility.

W. D. Terry: You teased us a couple of times with comments about the failure of your assumption that this is really nonspecific, but you haven't really said anything. Are you going to open up and tell us?

Weiss: My main reason for hedging now is the fact that Dr. P. Minden is spending a year with us in our laboratories and we shall have to await the results of his studies. I am impressed by some of his already reported findings showing specific antigenic cross-reactivity, quite well documented serologically, between mycobacteria and a variety of other taxonomically very distant organisms. Perhaps such cross-reactivity also includes certain tumor cells, but this is by no means clear to us yet.

So, it is a teasing proposition, and I just want to play it safe and suggest that there may be an element of specificity to what seems to be a nonspecific situation.

A. Goldin: I'm not clear, Dr. Weiss, about the synergism between MER and radiation and the extent to which you believe these are independent actions as opposed to protection which is now permitting the radiation to give you therapeutic effect.

Weiss: I don't know what the mode of action is, but let me summarize several observations.

With regard to already established, fairly large tumors, MER by itself has virtually no therapeutic effect. MER together with therapeutic irradiation or chemotherapy, or specific immunization with irradiated tumor cells, *can* have a very strong effect, even where those other therapies by themselves are only marginally effective or inactive.

With regard to the simulated metastases and simulated local-recurrence systems, MER by itself *is* often effective but more commonly, and to a larger extent, efficacy is clear-cut if there is chemotherapy; or, with regard to local recurrence, in situ irradiation superimposed on the MER treatment is extremely efficacious.

I tend to think that there may be a common denominator between irradiation and chemotherapy, which by destroying tumor cells makes available tumor-associated antigens and specific immunization. This conjecture may be wrong. The facts are that either of these 3 types of combined treatment induces a much stronger effect by MER and almost always prevents the development of enhancement.

T. Borsos: I'd like to make two comments. One of the critical things about experimenting with mice is that one has to be very careful what strains one uses, because different strains may respond very differently. In addition, mice may have difficulty with polysaccharide-type antigens, for mice are incapable of handling large amounts of polysaccharides.

The other comment I'd like to make is on the relationship between BCG and tumor cells. As so many times happens in science, the same thought occurs to different people at the same time. Thinking along the lines Dr. Weiss mentioned, we found some evidence a few months ago that line-10 tumor cells and BCG may indeed have a slight, but significant, antigenic cross-reactivity. This was detected with the C1 fixation and transfer test, a very sensitive complement-fixation test. The evidence is such that one cannot dismiss the idea that there is cross-reaction between line-10 tumor cells and BCG.

Weiss: I demonstrate my ignorance by not knowing that you have already shown this.

Borsos: We have not published these results. We are still working on this problem.

Weiss: I understand several workers at this meeting in England reported that enzymatic treatment of tumor cells reveals some such common antigens.

With regard to strains of mice, we have done experiments so far in 8 different inbred strains, and I have not found any impressive strain dependence for the effect.

With regard to other species, in a variety of immunologic stimulation models, MER has been given to rabbits, guinea pigs, mules, and men, and again activity was fairly broad in all of these species. I also note that the parameters of stimulation of cellular responsiveness of guinea pigs to haptene protein conjugates and of mice to allogeneic cells are similar to the parameters of the tumor-protective effect in mice.

Comparative and Combined Effect of BCG and Neuraminidase in Experimental Immunotherapy[1,2]

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SUMMARY—The total immunospecific regression of firmly established 3-methylcholanthrene (MCA) fibrosarcomas could be induced in syngeneic mice by inoculation of living tumor cells treated in vitro with *Vibrio cholerae* neuraminidase (VCN) plus mitomycin C. Multiple injections of such vaccines were more effective than a single inoculation. By comparison, BCG injected either directly into the tumor nodule or subcutaneously into sites distant from the tumor reduced the growth rate but rarely induced the total disappearance of the tumor. The use of neuraminidase-treated tumor vaccines was far more efficient than the use of BCG in inducing the regression of MCA fibrosarcomas. The rejection of the established MCA fibrosarcomas by VCN-treated tumor cells was augmented by the simultaneous injection of BCG. The maximum effect was obtained by combining the injection of VCN-treated cells with the injection of BCG at the same site.—Natl Cancer Inst Monogr 39:57–65, 1973.

A NUMBER of methods are available for inducing tumor-specific transplantation immunity against syngeneic tumors either before the tumors are inoculated or before the tumors have grown to discernible size. These methods include the injection of subthreshold doses of living tumor cells, irradiated cells, intradermal tumor cells, or cell-free extracts of tumor cells or the early excision of the growing tumor. Only 2 immunologic techniques, however, have successfully induced the regression of firmly established, growing, solid tumors in syngeneic hosts: *a*) the intralesional injection of living BCG organisms (*1, 2*) and *b*) the inoculation of living tumor cells treated in vitro with *Vibrio cholerae* neuraminidase (VCN) (*3, 4*). In the last technique, the total regression of firmly established 3-methylcholanthrene (MCA) fibrosarcomas was induced in syngeneic C3H/HeJ mice by inoculation of living tumor cells that had been treated with VCN. Regression could not be induced with cells treated with heat-inactivated VCN or cells incubated with VCN in an excess of competitive feedback inhibitor of the enzyme

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(sialic acid). The regression induced by VCN-treated tumor cells was immunospecific and could be induced only with VCN-treated cells identical in type with the growing tumor (*3, 4*). These findings combined with other evidence (*5–7*) strongly suggested that the result was due to an increased immunogenicity of the VCN-treated tumor cells.

The present experiments compare the effects of BCG, VCN-treated tumor cells, and the combination of BCG and VCN-treated tumor cells on the growth of firmly established tumors. The basic experimental design involved the inoculation of adult female C3H/HeJ mice with a predetermined number of untreated syngeneic tumor cells. When the tumor had become firmly established, BCG and/or tumor cells which had been incubated with VCN were injected. The effect of these secondary challenges on the growth of the established tumor was then determined.

MATERIALS AND METHODS

Mice.—C3H/HeJ adult female mice, 8–16 weeks old, were obtained from The Jackson Laboratory, Bar Harbor, Maine. They were housed in covered plastic cages, 10 or less per cage, and provided with tap water and a standard laboratory diet (Purina Chow Checkers) ad libitum.

Tumors.—Two MCA fibrosarcomas (MC-42, MC-43) were induced by the intramuscular injection of 0.5 mg MCA dissolved in 0.1% trioctinoin into the hind legs of C3H/HeJ females. The immunogenic characteristics of this tumor were thoroughly reported by Haywood and McKhann (*8*). The tumors have been serially transplanted in syngeneic female mice without any evidence of antigen modification or deletion. In unimmunized C3H/HeJ females, an inoculum of 120 MC-42 cells or 1,280 MC-43 cells will kill 50% of the recipients (*8*). Spontaneous regression does not occur. The tumors, specific for the strain in which they arose, will not grow in mice of different H-2 histocompatibility types. Haywood and McKhann demonstrated that these tumors are weakly immunogenic; i.e., inoculation of the tumor followed by amputation leads to tumor-specific resistance against subsequent inoculation (*8*). The MC-42 tumor, however, does not immunize against MC-43 or vice versa.

Sterile tumor cells for inoculation were prepared from tumors (1.0–1.4 cm in diameter) growing in C3H/HeJ females 3–4 weeks after inoculation. Cells were pressed through stainless-steel screens in medium 199 (M199) (Grand Island, N.Y.) without addition of trypsin. Cell clumps were then allowed to settle, and the supernatant single cells in suspension were washed 3 times in M199 and counted in hemocytometers. Viability was determined by trypan blue exclusion (*9*) before injection into the subcutaneous tissue of the lateral posterior flank of recipient C3H/HeJ females. The mice were inspected daily to determine the day of initial appearance of a palpable tumor. The largest diameter of the growing tumor was measured by calipers 3 times weekly, and the day of death was also recorded.

Neuraminidase.—VCN obtained from General Biochemicals, Chagrin Falls, Ohio, contains 500 U of enzyme/ml. (One unit of activity is equivalent to the release of 1 μg of *N*-acetyl neuraminic acid from a glycoprotein substrate at 37° C in 15 minutes at pH 5.5.) VCN is inactivated by being heated to 65° C for 30 minutes or 100° C for 10 minutes. Sialic acid is released from cell surfaces of normal and malignant cells by VCN without affecting viability (*10–15*). VCN releases about 250 nmoles of sialic acid from 10^9 MC-42 tumor cells used in these experiments (Ray, P. K., unpublished observations). Tumor cells in M199 were incubated for 1 hour at 37° C with 25 U VCN/ml 10^6 cells, with heat-inactivated VCN or with M199. Viability was confirmed (*9*), and the cells were washed 3 times before injection as a challenging inoculum into tumor-bearing hosts.

Mitomycin C.—Obtained from Sigma Chemical Company, St. Louis, Missouri, mitomycin C was incubated with tumor cells in a concentration of 25 μg/ml/10^6 cells in the same incubation mixture with VCN or heat-inactivated VCN. We previously demonstrated that the mitomycin C did not adversely affect the efficacy of VCN-treated cells to induce immunospecific regression of firmly established MCA fibrosarcomas in C3H/HeJ mice (*3*). Mitomycin C treatment of MC-42 or MC-43 tumor cells does not increase the uptake of trypan blue by such tumor cell suspensions but effectively prevents the growth of palpable tumors from the treated cells (*3*).

BCG.—*Mycobacterium bovis* strain BCG was obtained from Research Foundation, Chicago, Illinois. The vaccine was composed of viable organisms without preservatives. It was provided in ampules of 15 mg of dry powder which was then diluted in 1.5 ml of sterile water and injected subcutaneously into the mice in a volume of 0.1 ml containing 1 mg BCG (approximately 10^7 live organisms).

Experimental protocol.—The basic experimental design was to inoculate adult C3H/HeJ females with 20,000 viable tumor cells subcutaneously in the lateral posterior flank. The tumors usually were palpable by day 8 and measured 0.5–1.0 cm by day 15 when challenges with BCG plus VCN-treated cells were started. One mg BCG (0.1 ml) was either mixed with the tumor cells (1:1) or injected alone into the tumor nodule itself, or into another site. Tumor cells were either mixed with BCG or injected alone into sites distant from the growing tumor. The effect of the BCG and/or VCN-treated tumor cells on the growth of the firmly established MCA tumors was then determined.

RESULTS

Effect of BCG Dose on Regression of Firmly Established MC-42 Tumors in C3H/HeJ Mice

Into the subcutaneous tissue of the left flank of syngeneic C3H/HeJ mice were injected 20,000 untreated MC-42 cells on day 0. Fifteen days later, when the tumor size ranged from 0.5 to 0.8 cm in diameter, the mice received injections of varying quantities of BCG in the subcutaneous tissue of the right flank, distant from the growing tumor mass. All doses of BCG > 0.001 mg significantly ($P \leq 0.05$) slowed the growth of the tumors (table 1). The greatest inhibition of growth was obtained at a dose of 1 mg BCG/mouse. In addition to slowing growth, tumor-bearing recipients of 1 mg of BCG lived longer than did untreated recipients ($P \leq 0.05$) Survival was not significantly prolonged at lower doses. The 10-mg dose of BCG was less effective than the 1-mg dose, although the tumor was still significantly smaller than that in the controls. The apparent shortening of life induced by 10 mg of BCG was not statistically significant ($P \leq 0.20$). None of the tumors firmly established when BCG treatment was initiated totally disappeared, and all tumors grew and ultimately killed the recipients.

TABLE 1.—Effect of subcutaneous inoculation of viable BCG organisms on growth of established MC-42 tumors in syngeneic C3H/HeJ mice*

BCG dose (mg)	Fraction of tumors regressing	Mean tumor diameter at day 40 (cm ± SE)	Mean day of death (± SE)
0.001	0/8	2.44±0.14	51.5±2.01
0.01	0/8	1.90±0.13	52.5±1.57
0.1	0/8	1.83±0.13	51.9±1.76
1.0	0/8	1.55±0.16	57.4±1.13
10.0	0/8	2.05±0.13	47.9±1.82
M199	0/8	2.83±0.12	51.6±2.17

* Single injections of BCG were given in the right flank 15 days after inoculation of 20,000 MC-42 cells into the left flank.

Comparative Effect of BCG Injected Into Tumor Nodule or at a Subcutaneous Site Distant From Tumor

Injected into the subcutaneous tissue of the left flank of syngeneic C3H/HeJ mice were 20,000 untreated MC-42 cells on day 0. At various intervals thereafter, single or multiple doses of 1 mg BCG were injected either directly into the tumor nodule or into the subcutaneous tissue at a site distant from the tumor nodule. The effect of these inoculations on the growth of the firmly established MC-42 tumor is indicated in table 2. Four facts can be discerned from this table: *a*) Injection of BCG directly into the tumor or at a site distant from the tumor inhibits the growth of firmly established MC-42 tumor. *b*) The inhibiting effect of BCG injection is diminished the longer treatment is withheld. *c*) The inoculation of BCG directly into the tumor nodule is somewhat more effective than inoculation of BCG into the subcutaneous tissue at a site distant from the tumor nodule. *d*) Six injections of BCG given on alternate days are more effective than a single injection. The most effective treatment in reducing the tumor size and prolonging life appears to be multiple injections of BCG directly into the tumor beginning as soon after the tumor appears as is possible. If 6 doses of 1 mg BCG are injected directly into the tumor nodule, an occasional tumor will disappear and the animal survives indefinitely. However, even if treatment is delayed for 30 days, BCG may significantly prolong life without reducing the size of the tumor mass.

Comparative and Combined Effect of BCG and/or VCN-Treated MC-42 Cells on Growth of Established MC-42 Tumors in Syngeneic C3H/HeJ Hosts

Into the left flank of recipient C3H/HeJ mice were injected subcutaneously 20,000 MC-42 tumor cells. Fifteen days thereafter, when the tumor nodules measured 0.05–1.0 cm in diameter, recipient mice were given injections of 1 mg BCG and/or 10^6 living MC-42 tumor cells which had been incubated either with VCN (plus mitomycin C) or heat-inactivated VCN (plus mitomycin C). The results are summarized in table 3. MC-42 cells in untreated animals uniformly developed into tumors which killed the recipients by 70 days. The subcutaneous injection of BCG into the opposite side 15 days after the primary tumor inoculum slowed the growth

TABLE 2.—Comparative effect of single* or multiple† BCG injections on growth of established MC-42 tumors in syngeneic C3H/HeJ mice

Interval between injection of tumor and treatment (days)	Number of BCG treatments* †	Site of BCG injections	Fraction of tumors regressing	Mean tumor diameter at day 40 (cm ± SE)	Mean day of death (± SE)‡
15	1	Intratumor	0/8	1.66±0.16	61.4±2.30
	6	Intratumor	1/8	1.19±0.16	65.9±3.76‡
	1	Subcutaneous	0/8	1.63±0.12	58.5±2.02
	6	Subcutaneous	0/8	1.56±0.08	63.0±2.15
20	1	Intratumor	0/8	1.81±0.15	62.0±2.00
	6	Intratumor	0/8	1.64±0.15	66.5±2.23
	1	Subcutaneous	0/8	2.14±0.17	56.8±2.17
	6	Subcutaneous	0/8	1.89±0.15	61.4±3.19
30	1	Intratumor	0/8	2.82±0.08	54.2±1.79
	6	Intratumor	0/8	2.65±0.12	59.9±2.59
	1	Subcutaneous	0/8	2.60±0.11	60.5±2.26
	6	Subcutaneous	0/8	2.44±0.12	68.3±3.64
—	M199	—	0/8	2.69±0.13	54.2±2.42

* 1 mg BCG 15 days after tumor inoculation.
† 1 mg BCG every other day for 6 doses.
‡ Mice whose tumors totally regressed are not included.

of the tumor slightly. Injecting 10^6 tumor cells exposed to heat-inactivated VCN did not affect the progressive fatal growth of the tumor. In contrast, injecting 10^6 VCN-treated cells markedly slowed the growth of the tumor and produced total regression of 2 of 17 firmly established, solid MC-42 tumors. Animals whose tumors totally disappeared survived indefinitely; life was slightly prolonged in those animals whose tumors did not totally disappear.

The simultaneous injection of BCG and tumor cells into separate sites distant from the primary growing tumor was then assessed (table 3, expts. 5–10). The simultaneous injection into separate sites of 1 mg BCG and 10^6 MC-42 cells which had been exposed to heat-inactivated VCN had no more effect on the growth of the tumor than did the injection of BCG alone. The simultaneous injection of BCG and MC-42 tumor cells which had been exposed to VCN led to the total regression of 16 of 56 tumors. The tumors remaining grew very slowly and life was prolonged. Animals whose tumors totally regressed survived indefinitely.

The site of BCG injection influenced somewhat the response of the tumors. If the BCG was injected directly into the tumor mass and the VCN-treated tumor cells injected elsewhere, only 1 of 14 tumors regressed, although tumor growth was severely inhibited. If the BCG was injected at a separate location from the injection of VCN-treated MC-42 tumor cells, or mixed with the cells, 15 of 42 tumors totally disappeared, and the animals survived indefinitely.

Effect of Multiple Doses of BCG and VCN-Treated MC-42 Cells on Growth of Established M-42 Tumors in Syngeneic C3H/HeJ Mice

Injected subcutaneously into the left flank of recipient C3H/HeJ mice were 20,000 MC-42 tumor cells. Fifteen days thereafter, when the tumor nodules measured 0.5–1.0 cm in diameter, recipient mice were given injections of BCG and/or 10^6 living MC-42 tumor cells which had been incubated either with VCN (plus mitomycin C) or heat-inactivated VCN (plus mitomycin C). The injections were repeated on alternate days until a total of 6 injections had been given. The results are summarized in table 4 and text-figure 1. They can be compared directly with those given in table 3 because the experiments were carried out simultaneously. Firmly

TABLE 3.—Combined effect of a single challenge with VCN-treated cells plus BCG on the regression of MC-42 tumors in C3H/HeJ mice

Experiment No.	Treatment of cellular challenge*		BCG challenge†			Fraction of tumors regressing	Mean tumor diameter at day 40 (cm ± SE)	Mean day of death (± SE)‡
	VCN	Inactivated VCN	Separate	Challenge	Intra-tumor			
1	No Challenge		—	—	—	0/18	2.45±0.09	56.2±1.5
2	—	—	+	—	—	0/21	1.83±0.14	54.7±2.2
3	—	+	—	—	—	0/18	2.33±0.09	55.6±1.4
4	+	—	—	—	—	2/17	1.11±0.13	65.6±2.4
5	—	+	+	—	—	0/22	2.15±0.09	52.4±1.8
6	+	—	+	—	—	7/21	0.97±0.13	68.2±2.4
7	—	+	—	+	—	0/22	1.93±0.07	54.7±1.7
8	+	—	—	+	—	8/21	0.89±0.12	69.5±3.2
9	—	+	—	—	+	0/14	2.23±0.17	63.8±1.8
10	+	—	—	—	+	1/14	0.98±0.15	68.5±3.4

* Challenging inocula consisted of a single injection of 10^6 MC-42 cells which had been incubated with 25 U/ml/10^6 cells of VCN or heat-inactivated VCN (plus mitomycin C). The challenge was given at a site distant from the tumor 15 days after tumor inoculation.

† BCG treatment consisted of 1 mg BCG injected at a site separate from the cell challenge, with the cellular challenge, or directly into the tumor mass.

‡ Does not include mice whose tumors totally regressed.

TABLE 4.—Combined effect of 6 challenges with neuraminidase-treated cells plus BCG on regression of MC-42 tumors in C3H/HeJ mice

Experiment No.	Treatment of cellular challenge*		BCG challenge†		Fraction of tumors regressing	Mean tumor diameter at day 40 (cm ± SE)	Mean day of death (± SE)‡
	VCN	Inactivated VCN	Separate	With challenge			
1	No Challenge		—	—	0/18	2.45±0.09	56.2±1.5
11	—	—	+	—	1/21	1.58±0.17	49.3±3.1
12	—	+	—	—	0/18	2.34±0.10	56.7±1.7
13	+	—	—	—	6/17	0.78±0.13	66.0±1.9
14	—	+	+	—	0/21	1.99±0.13	53.0±2.0
15	+	—	+	—	10/21	0.80±0.13	64.3±2.9
16	—	+	—	+	0/21	1.97±0.14	54.3±2.0
17	+	—	—	+	14/21	0.55±0.11	70.1±6.3

* Challenging inocula consisted of 6 injections of 10^6 MC-42 cells which had been incubated with 25 U/ml/10^6 cells of VCN or heat-inactivated VCN (plus mitomycin C). The first challenge was given 15 days after tumor inoculation and subsequent challenges at 2-day intervals.

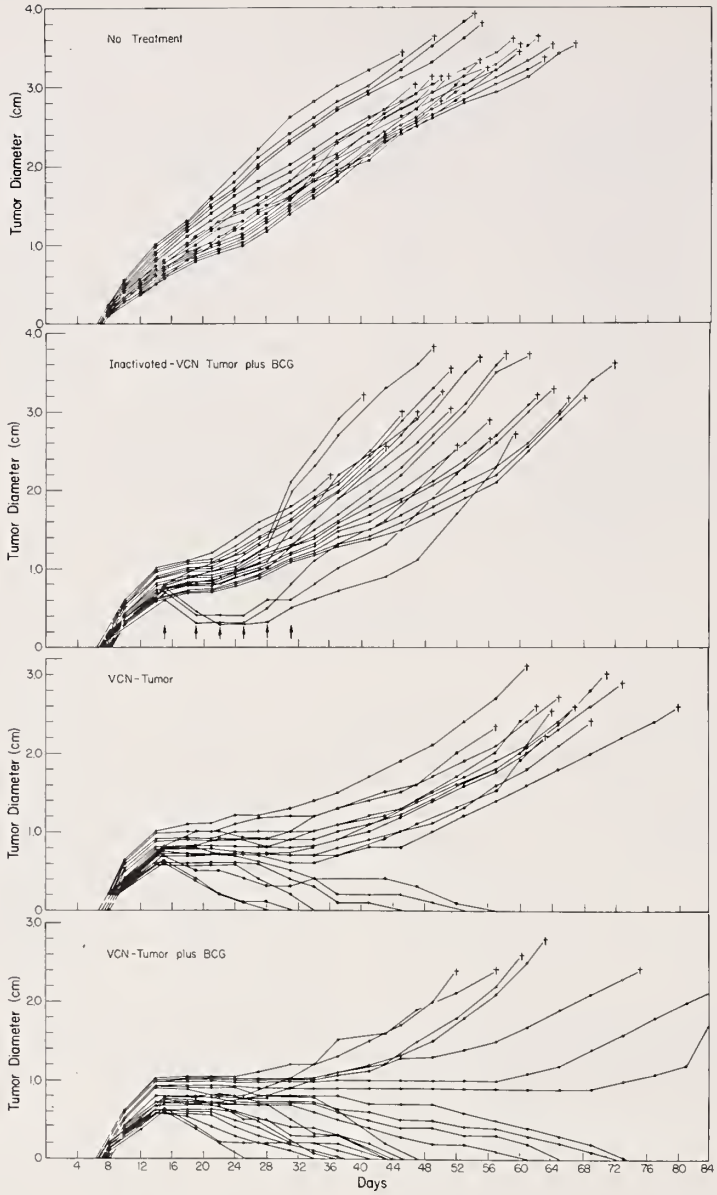
† BCG challenge consisted of 1 mg BCG injected at a site separate from the cell challenge or with the cell challenge.

‡ Does not include mice whose tumors totally regressed.

established tumors never regressed in animals who received no treatment or in animals who received tumor cells which had been incubated with heat-activated VCN, even if those injections were given simultaneously with, or at the same site as, BCG inoculations. BCG by itself induced the regression of only 1 of 21 tumors. In contrast, 6 injections of VCN-treated tumor cells induced the total regression of 6 of 17 tumors. When VCN-treated cells were given simultaneously with BCG injections, 24 of 42 tumors totally regressed. When the tumors did not totally regress, their growth was markedly slowed and the life of surviving animals prolonged. In this experiment, combining BCG with VCN-treated tumor cells at the same injection site appeared slightly more effective than giving the injections simultaneously, but at different sites.

Immunospecificity of Tumor Regression Induced by BCG and VCN-Treated Tumor Cells

C3H/HeJ mice were inoculated subcutaneously in the left flank with 20,000 MC-42 cells and in the right flank with 20,000 MC-43 cells. Twelve days later, recipients were challenged with 1 mg BCG and/or 10^6 tumor cells which had been exposed to VCN or heat-inactivated



TEXT-FIGURE 1.—Combined effect of VCN-treated MC-42 tumor cells and BCG injected simultaneously into mice bearing firmly established MC-42 tumors: Untreated tumors (*first graph*) killed animals in about 2 months. Six injections of BCG injected on alternate days beginning 15 days after tumor transplant slowed tumor growth. The simultaneous inoculation of BCG plus MC-42 tumor cells which had been exposed to heat-inactivated VCN had no greater effect than did the inoculation of BCG alone (*second graph*). Six injections of 10^6 VCN-treated MC-42 cells produced the regression of approximately 1 of 3 growing tumors (*third graph*). The simultaneous injection of BCG and 10^6 VCN-treated MC-42 cells led to the total rejection of 2 of 3 of the firmly established MC-42 tumors (*fourth graph*).

VCN. Alternate groups of mice were challenged only with MC-42 cells (and/or BCG) or MC-43 cells (and/or BCG). The results are shown in table 5.

The MC-42 tumors in the left flank of mice challenged with VCN-treated MC-42 cells grew less well than MC-42 tumors in animals challenged with MC-43 cells. The opposite effect

TABLE 5.—Immunospecificity of the combined effect of a single challenge with neuraminidase-treated MC-42 or MC-43 cells plus BCG on the regression of MC-42 and MC-43 tumors in C3H/HeJ mice*

Challenging tumor treated with VCN + M†	BCG with challenging tumor	Fraction of tumors regressing		Mean tumor diameter at day 40 (cm ± SE)		Mean day of death (± SE)
		MC-42	MC-43	MC-42	MC-43	
MC-42	—	2/8	0/8	0.46±0.47	2.30±0.06	37.6±1.9
MC-43	—	0/8	3/8	2.24±0.05	0.54±0.24	40.1±2.5
None	+	0/9	0/9	1.50±0.13	1.56±0.15	48.7±2.3
MC-42	+	4/8	0/8	0.38±0.23	1.42±0.09	47.3±2.6
MC-43	+	0/9	4/9	1.44±0.12	0.59±0.24	46.2±2.5
None	—	0/9	0/9	2.12±0.08	2.38±0.06	39.0±2.3

* Challenging inocula were given 12 days after the MC-42 tumor had been injected on the left and the MC-43 tumor injected on the right.
† Challenging inocula consisted of 10^6 MC-42 or MC-43 cells inoculated with VCN and mitomycin C.

was noted in animals challeged with MC-43 cells exposed to VCN. This immunospecificity of response has previously been described (*4*). No such immunospecificity was seen in animals challenged with BCG without tumor cells. Growth was slightly inhibited in both tumors, and the life of the tumor-bearing mice was slightly prolonged.

When BCG was combined with the VCN-treated cells, the immunospecificity of the reaction was not affected. BCG plus VCN-treated MC-42 cells induced only the regression of MC-42 tumors in animals bearing both MC-42 and MC-43 tumors. The MC-43 tumors progressed and killed the animals. Life was slightly prolonged in these animals, as it was in the animals treated with BCG alone. Conversely, in mice bearing bilateral tumors treated with BCG plus MC-43 tumors exposed to VCN, only MC-43 tumors were induced to regress. The MC-42 tumors grew only slightly slower than normal and at an identical rate as that in animals treated with BCG alone. The animals all died of one tumor, while rejecting the other.

DISCUSSION

Those substances which nonspecifically increase resistance to syngeneic tumors were recently reviewed by Yashphe (*16*). BCG organisms have been most commonly employed, although *Corynebacterium parvum, Bordetella pertussis,* streptococci, serratia, bacterial toxins, DNA digests, and polyanionic substances have all been found to stimulate nonspecific resistance to tumors (*16*).

If administered several days to several weeks before the transplantation of the tumor, viable BCG organisms increase the resistance of animals to the transplantation of syngeneic tumors. In addition, the prior administration of BCG delays the appearance (or decreases the incidence) of carcinogen-induced tumors, radiation-induced tumors, and even spontaneous tumors in mice (*16*). Mathé et al. noted that BCG plus irradiated tumor cells will immunize mice to the nonspecific L1210 leukemia even after inoculation of viable tumor cells (*17*). BCG also prolonged the survival of patients with acute lymphoblastic leukemia after chemotherapeutic remission (*18*). Morton et al. noted that the intralesional injection of BCG into melanoma nodules induced regression of melanomas in man (*1*), and Zbar and Tanaka found that BCG injected directly into syngeneic guinea pig hepatomas induced their regression (*2*). Although BCG can be utilized in the phenolized form and in the form of methanol-extracted residue (*16*), Zbar and Tanaka believed that the effect of BCG is magnified by the use of viable organisms, by direct contact of the BCG with the tumor, and by prior sensitization of the animal to BCG (*2*). Similarly, Morton et al. found melanomas to respond to intratumor injections of BCG only in patients who became sensitized to the BCG (*1*).

Our previous results confirmed that the administration of BCG to C3H/HeJ mice bearing firmly established, solid MC-42 tumors temporarily slowed tumor growth. The injections were more effective than single injections (possibly due to sensitization of the mouse to the

BCG), but regression could not be induced after the tumor had been present for prolonged periods and had become rather large (*19*). We also demonstrated that the total rejection of firmly established MC-42 or MC-43 fibrosarcomas by syngeneic C3H/HeJ mice could be induced with challenging inocula of living tumor cells which had been treated in vitro with VCN (*4*). Regression could not be induced with cells treated with heat-inactivated VCN plus cells incubated with VCN and an excess of competitive feedback inhibitor of the enzyme (sialic acid). Regression induced by VCN-treated tumor cells was immunospecific and could be induced only with VCN-treated cells identical in type with the growing tumor (*4*). We also produced immunospecific regression of syngeneic MCA fibrosarcomas by the direct intratumor injection of VCN (*20*). Of the methods previously investigated, the injection of VCN-treated tumor cells into tumor-bearing mice was by far more effective than either the intralesional injection of VCN or the intralesional injection of BCG.

BCG and VCN-treated tumor cells probably exert their respective tumor-inhibitory effects via different mechanisms. BCG may act to induce a local inflammatory response, thereby attracting immunoreactive cells to the site of the tumor and, in turn, increase the contact between the immunoreactive cells and the weakly immunogenic tumor (*21*). It therefore is more effective when the recipient has been preimmunized and when injection is directly into the tumor tissue.

VCN acts on the cell surfaces to lyse the 2-3′, 2-6′, and 2-8′ glycosidic linkages between sialic acid residues and the mucopolysaccharides of the cell surface (*22, 23*). Removing these sialic acid residues not only may remove a stearic hindrance to antigen perception but may reduce the negative charge of the cell (*22, 23*), increase cell deformability (*24*), and increase the susceptibility of the cell to phagocytosis (*23, 25*). All of these factors would be expected to increase the availability of tumor-specific antigenic sites on the cell surface to immunoreactive cells in the host (*7*). A considerable amount of data supports the hypothesis that cells exposed to VCN are rendered more immunogenic. Normal lymphoid and embryonic cells exposed to VCN and injected in small nonimmunogenic doses into allogeneic recipients will sensitize those recipients (*23, 25*). Human peripheral blood lymphocytes exposed to VCN and incubated with normal allogeneic lymphocytes in mixed lymphocyte cultures (MLC) will double the rate of DNA synthesis in the allogeneic cells compared with the normal MLC response (*5*). Schlesinger and Amos (*26*) and Kassulke et al. (*27*) have even demonstrated the unmasking of various antigens on cells treated with VCN, although this may not be true for all antigens (*13, 14*). Even immunosuppressed recipients can be immunized by cells treated with VCN (*28*). Watkins et al. recently showed that human lymphocytes will transform in response to VCN-treated autologous tumor cells in tissue culture (*29*). That such a variety of normal and malignant cells can be rendered more immunogenic by treatment with VCN suggests that VCN acts nonspecifically to render cells more susceptible to immunologic processing by the recipient. Such a process is analogous to changing the immunogenicity of a hapten by changing the characteristics of the carrier molecule.

Whatever the mechanism, the present immunotherapy model can easily serve as a model for the study of other variables in cancer immunotherapy, i.e., their combination with surgical excision, chemotherapy, and radiotherapy. Such a model also provides a reasonable basis for experimental cancer immunotherapy in man where BCG is already in use (*1, 18*). Since mitomycin C treatment of tumor cells prevents the growth of tumor, one need not fear the growth of reinjected viable VCN-treated tumor cells into the human recipient. Such a model may provide the basis of a VCN and mitomycin C-treated tumor vaccine which can be used in patients in whom conventional therapy has been shown to be ineffective.

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DISCUSSION

G. B. Mackaness: Are there any questions for Dr. Simmons?

R. Powles: Did you try prophylactic experiments in those elderly mice by giving them repeated injections of tumor cell plus BCG to see if you could prevent them from getting tumors?

R. L. Simmons: We are doing those experiments now. So far, among those mice, about 50% have died in 6 months. Only mice who got BCG died. The neuraminidase didn't seem to increase the toxicity of the BCG, and neither the neuraminidase nor the BCG, which was given on the back of the mice, seemed to affect the

incidence of tumors in the elderly mice.

We also have a cohort group of young C3H mice that we're going to to follow for several years; we don't know what effect prolonged treatment will have. Only 1 mg BCG has been used—which is really not a fair dose, considering that we know that there's a marked dose response.

W. D. Terry: Are the 2 effective systems both in C3H mice?

Simmons: Except for the B16 melanoma in the C57BL/6 mice.

Terry: So there is a system in C57BL which works in a comparable manner and gives the same approximate statistics?

Simmons: In the 2 sets of experiments we've done involving 20 mice in both groups, it seems to work the same.

J. W. Kreider: Have you tried BCG in the B16 melanoma?

Simmons: No. We have used only neuraminidase-treated tumor cells.

A. Goldin: What do you do with the spontaneous mammary tumor? Did you inject the neuraminidase itself into tumor cells?

Simmons: The first set of experiments involved taking a tumor out, treating the tumor with neuraminidase in single-cell suspension, and injecting this tumor back into the mice. Because this seemed to work, it seemed ridiculous to excise the tumor; therefore, we ran a series of experiments in which we injected the neuraminidase, 50 U/day, directly into the 3-methylcholanthrene (MCA)-induced tumor. This also led to immunospecific regression of the tumor cells; so if we had an MC-42 tumor on the left and an MC-43 tumor on the right and treated only the tumor on the left with intratumor inoculations of neuraminidase, only the left tumor went away and the right tumor continued to grow and kill the animal. If the tumors were identical on the left and on the right and we gave injections to the tumor on the left, which went away, then the tumor on the right also went away.

Therefore, because of the possible individual immunospecificity of each of the spontaneous tumors, we believed the only reasonable model, without partial excision of every tumor, would be to inject the neuraminidase directly into the tumor. By analogy only, we hope that their regression is an immunotherapeutic effect and not one due to nonspecific causes.

Goldin: We did some early experiments with just chemotherapy of spontaneous mammary tumor. The animals had many tumors. We found that, in the same animal, some tumors responded whereas other didn't Have you worked with animals having multiple tumors?

Simmons: Not with multiple spontaneous tumors. We have done several experiments with multiple transplanted tumors of various sizes. For example, we have grafted the MC-42 tumor on day 0 and the MC-43 tumor on day 15. We then treated the MC-42 tumor (or MC-43 tumor) on day 30 and found out which set of tumors would go away. The smaller specific tumors regressed in the animals who had a total small mass whether immunologically specific or nonspecific.

Thus the specific tumor being treated had to be reasonably small and, if the nonspecific tumor was very large (>2 cm in diameter), immunotherapy wouldn't work.

J. C. Kennedy: Have you injected neuraminidase into secondary tumors?

Simmons: We treated 3 secondary tumors with neuraminidase and BCG; like the treated primary tumors, they stopped growing.

L. Nathanson: Have you used *Vibrio cholerae* neuraminidase (VCN) with any extract or membrane fraction of tumor cells?

Simmons: No.

F. C. Sparks: In a slightly different experiment with the tumor that you sent to me, we put the VCN in the axilla as a control in tumors that were in just 1 leg. To our surprise, we got a systemic effect in regression of the tumor in the leg, a little bit different from that seen with your contralateral tumor.

Simmons: We have done that experiment and found no effect. Haven't you also seen some enhancement with neuraminidase in some systems, Dr. Sparks?

Sparks: Yes.

Simmons: Possibly, in the experiments using spontaneous tumors, we were enhancing one set of antigens and regressing another set of antigens on the same tumor cell.

Terry: A specific question: On day 0, suppose you have the MC-42 tumor on the left and one on the right, and you inject the VCN only into the left tumor, which is one of the tumors that goes away. Does the tumor on the right also go away?

Simmons: If both tumors are immunologically identical.

Terry: How many different deposits of MC-42 tumors can you put in and still get regression of the tumor? What kind of tumor load will the system handle? Clearly we all find that there is a limit in the manipulation.

Simmons: There seems to be a definite quantitative limit to the amount of tumor which will regress in response to immunotherapy.

D. W. Weiss: We carried out an experiment some years ago in Berkeley. We injected intraperitoneally different amounts of methanol-extraction residue (MER) or living BCG into young C3H mice once a month for 4 months during the first half year of life. We then left the animals alone and put them into a normal breeding colony. Saline controls were used. Animals were given a total of 0.4 mg MER. Spontaneous mammary carcinoma onset was markedly retarded. As we tripled the dose and again tripled it, the protective effect was lost and there was a move toward enhancement. Living BCG given in fairly large amounts in this system strongly tended toward enhancement. The parameter of dosage of nonspecific stimulators must be considered in any of the effects ascribed to such agents.

Mackaness: Dr. Simmons, have you looked at the effect of varying the dose of BCG? These older animals with

spontaneous tumors, tend to have "BCG-osis." Could the dwindling immunocompetency of the older animal be a factor? A lower dosage of BCG in the older groups might be beneficial.

Simmons: You understand that's a very long-term and difficult experiment, for you would have to hunt for spontaneous tumors for months. Therefore, we don't have a dose response.

In the MCA-induced tumors, however, we have determined the dose effect of a single shot of BCG directly into the tumor. In this system, 10 mg BCG is far worse than 1 mg which is better than 0.1 or 0.01 mg.

Terry: Are you going to tell us, or at least speculate for us on how, when you inject the enzyme into the tumor, it does anything?

Simmons: I don't know exactly what it does. I presume that VCN acts as an enzyme on the tumor cells in vivo. With just a few injections of neuraminidase, nothing happens. However, if you inject neuraminidase every day for 2 months, tumors will totally regress and necrosis is absent at the site of the tumor itself. If you give the neuraminidase elsewhere in the animal, tumors will not regress. This is a very expensive experiment. Neuraminidase costs $10 for 500 U. This means $10/10 mice/day for life.

Terry: What is the mechanism?

Simmons: Neuraminidase has many effects on the cell surface: It changes the cell's charge, its deformability, and its ability to be phagocytized. Almost every protein treated with neuraminidase will show an increase in immunogenicity. In general, VCN increases the immunogenicity of all the antigens on a cell surface.

In addition, S. A. Rosenberg et al. and D. B. Amos et al. have shown (and we have also confirmed) that treating cells with neuraminidase unmasks new determinants for which preformed antibodies exist—probably in the same animal—but certainly in heterologous species.

My current thinking is that, if you expose new antigens and return the cells to the same animal, the animal can process this new antigen more readily. Thus, by processing the previously recognized antigens, the new antigens on the same cell are rendered more immunogenic.

Unidentified speaker: In reference to Dr. Terry's earlier question about how many sites can be handled in the guinea pig experiment: In the assessment of tumor-specific immunity, the limitations of immunity seem to be related more to total-body tumor burden than to the number of sites in which that amount of tumor is distributed. If the same number of cells could be rejected at a single site, it didn't matter whether they were in 1 site or 10 sites. But if there were too many cells at 1 site and they were spread out in 10 sites . . . so it's total-body tumor burden, I think, probably more than number of sites.

B. H. Waksman: To add to what Dr. Simmons just said, people for a number of years have tried different devices for modifying surface antigens on tumor cells. This neuraminidase technique is a very nice one, evidently. Some years back B. G. Arnason et al. found that iodoacetate could endow tumor cells with a new antigenicity and enable these cells to be rejected in situations in which they would otherwise not be rejected.

The same was done, in a very elegant series of studies, by Lindeman working with influenza virus and ascites tumor cells. He found that virus which grew in the cytoplasm of tumor cells became extremely antigenic for tumor; i.e., a tumor which would not normally be rejected was now easily rejected by animals given injections of cells coated with virus antigen. So introducing new antigenic determinants in relation to the tumor cell antigens is a powerful way of getting better immune response to the tumor antigen itself.

Simmons: Remember this: VCN is a different enzyme than the influenza virus neuraminidase, for it has a different substrate.

Waksman: The flu wasn't acting in terms of neuraminidase; its antigenicity was the significant property.

Simmons: But people should know that.

R. B. Herberman: Just to elaborate on the question of mechanism, I am a little surprised that neuraminidase, which has a pH optimum way below the physiologic pH, should be as effective as it is in this in vivo inoculation. How do you reconcile this with the type of observation that J. G. Bekesi made, for instance?

Simmons: Dr. Bekesi made similar observations, but he used a different pH when he treated a cell. But Rosenberg et al.[1] showed that neuraminidase at neutral pH is 75% active in removing sialic acid. In fact, everyone has shown that neuraminidase, despite its pH optimum of 5.5, is very active at neutral pH.

M. A. Chirigos: In the MC-42 or MC-43 system in which you divided your therapy and got cures, did those animals so treated withstand a challenge? And if so, for how long did they withstand it?

Simmons: We haven't tested that. We gave the animals a million cells 30 days after all the rest of the animals had died. And those animals always withstood a tumor inoculum of a million cells. Thirty days later, they were given a million cells of the opposite tumor, and 100% of those all died. Therefore, they were highly immune to the specific cure, and they were highly susceptible to the opposite tumor.

S. R. Rosenthal: I would like to relate some experiences that G. Barski has had in Paris with regard to tumor size and the immune system in general. Dr. Barski found that, if he took the macrophage from the peritoneum of mice given certain tumors, at an early time those macrophages would be cytotoxic to the tumor cells. When the tumors grew to about 10 mm in about 10 or 12 days, they lost that capacity and lost it indefinitely after reaching that size. In other words, they lost their immune capacity. This is a very graphic representation of what everybody has been talking about: There's actually

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[1] Rosenberg SA, Plocinik BA, Rogentine GN Jr: "Unmasking" of human lymphoid cell heteroantigens by neuraminidase treatment. J Natl Cancer Inst 48:1271–1276, 1972.

an immune suppression by the tumor after it reaches a certain size.

Simmons: We've done some excision experiments which suggest it is not just that. We almost totally excised the tumors at 2 cm so that there was much less tumor there —and this was not a widely metastasized sarcoma—and then challenged the tumors with neuraminidase-treated tumor cells. Within 3 days, the tumors regrew to their previous size and killed the animal; however, in 1 animal the tumor totally regressed, perhaps because we totally removed the tumor as well. Therefore, it cannot be the tumor size alone (although this is important); some irreversible change must occur in the mice which is correlated with tumor size.

Rosenthal: That is true. But you recall the first experiment some time ago in which it was found that, if you take the spleen cells in a rat after the tumor is still a large tumor, they will not inhibit the growth of similar cells in another animal. But if you take the tumor out, and then 3 weeks later take these spleen cells, then they will be inhibitory. In other words, they recouped. They still have the memory for the immunity, but at the time the tumor is large their capacity to act is suppressed.

Unidentified speaker: It's important to add to this discussion that Barski in his work showed that not only were the peritoneal cells not protected in the tumor-bearing animal but also the target cells grew much better than was anticipated in controls.

R. A. Phillips: The preceding papers indicate that BCG, MER, and VCN have certain things in common: They induce regression in some cases and in others, enhancement. I'm sure everybody would like to avoid enhancement, but could we come to any generalization regarding the factors influencing enhancement in these often observed cases?

Mackaness: Has anybody seen any common thread of conditions that lead to enhancement as opposed to regression?

Weiss: They say fools rush in where angels fear to tread, so I'll tread very lightly. In a variety of models, especially in a guinea pig model in which we looked for effects of MER on cellular versus humoral immunity against otherwise nonimmunogenic haptene-protein conjugates, the one generality that could be made, and which seemed to apply at least to some extent to tumor systems, was: If the amount of the nonspecific stimulator is small —and even more critically—if the amount of the specific antigen to be stimulated against is small, there is a definite bias toward cellular activity.

If, on the other hand, the amount of antigen and/or the amount of stimulator is large, the bias seems to be toward humoral immunity. I cannot say this holds true down the line, but it holds true in several of the systems we've looked at.

In addition, combined treatment schedules (MER plus irradiation, or chemotherapy) seem to result either in heightened resistance or in little change over the controls, but not in accelerated tumor growth; in cellular terms, combined treatment may depress the appearance of blocking antibodies. Again, I am not at all sure we can generalize, but in some systems this seems to be so.

Mackaness: In our own limited experience using line-10 tumor in strain-2 guinea pigs, we found that some doses of BCG tended to enhance. But we have also found that, if we gave over-doses of BCG, we could in fact destroy the immunologic apparatus. And that in itself is obviously not in the interest of the subject.

H. J. Rapp: Is that in the guinea pig?

Mackaness: No, in the mouse.

Rapp: I don't think you'll find that in guinea pigs.

Mackaness: That's very possibly true. It's a point well worth remembering that mice, guinea pigs, and man differ from each other in many ways.

Rapp: There is evidence that in some ways guinea pigs immunologically may more closely resemble man than other laboratory animals. For example, rats, mice, and hamsters handle BCG differently than guinea pigs. One reason for starting our work in tumor immunity with guinea pigs 10 years ago was that the skin reactivity of guinea pigs and man is similar; mice and rats are quite different.

Waksman: Excuse me. If I may say so, that's not really true. Rats get extremely good skin reactions. They're just as large as guinea pig skin reactions. It's a matter of using the dosage to which rats respond. For years it has been that guinea pigs resemble man with regard to delayed sensitivity and that rats and mice do not. But I really don't think that's true.

Rapp: That may or may not be true. The other point is that guinea pigs and humans seem to resist polycyclic hydrocarbon carcinogenesis.

U. Saffiotti: Again we can't generalize.

Mackaness: We are entering upon a topic that is to be discussed this afternoon: the adequacy of the models we're using for study of cancer and the effect of BCG.

L. Chedid: I have one question relevant to the assay system introduced by Dr. Rapp and his associates: They are using strain-2 inbred guinea pigs which, according to my information, elicit weak cell-mediated immune responses and strong humoral responses. This is rather baffling.

Mackaness: Can Dr. Chase answer that question?

M. Chase: I'll answer it indirectly. Strain-2 guinea pigs do not sensitize well with mycobacteria to give tuberculin responses, but they handle mycobacteria bodies rather well as adjuvant. So you have a double-barreled thing. Now, I haven't got the rest of your question.

Chedid: I thought they gave good humoral responses.

Chase: They give very good antibodies.

Chedid: This seems paradoxical.

Rapp: No, because cellular immunity is not lacking. As a matter of fact, we could detect the skin reactivity of tumor-specific antigens very nicely in strain-2 guinea pigs.

INTRODUCTION: AFTERNOON SESSION

I must say at the outset of this afternoon's session that I am going to find it very difficult to follow Dr. Mackaness as chairman. As a means of security, I would at least like to begin my brief introductory remarks by leaning on one of Dr. Mackaness's last statements, which was the truism that mice, guinea pigs, and man differ from each other. This essentially sets the tone for what is to be our discussion this afternoon.

According to our program, we should ask the basic question of animal test systems: Do they serve as models for human immunotherapy? This question is of course only a part of a much larger and more universal one: To what extent can one extrapolate from species to species, especially when phylogenetic distance between them is as great as is the gap between man and the usual species of laboratory animals?

In biologic terms, this question is almost ludicrous. As all of us know, it is very difficult to isolate and examine a single variable intrinsic to host and parasite within a given model, even when one deals with an inbred strain. When one considers the jump from one class or order to another, the task of comparison and extrapolation begins to seem impossible. Yet, the task is inescapable because we have no choice but to use animal models despite what seems to be at times a labor of Sisyphus.

I would, therefore, suggest that our theme this afternoon be the question so rephrased as to remain in the realm of empirical reality: What frameworks, or what basic conditions, of animal experimentation can be designed that will minimize the inherent deficiency, if not illogicality, of animal models for a human situation? This would be putting the matter much more conservatively and more precisely!

It would seem to be clear at first sight what *cannot* be done in an attempt to construct animal models as a basis for human study: This is to work in one or a few isolated systems, and in such isolation to pose specific queries based on the assumption that direct extrapolation is valid. For example, one cannot conclude from the observation that the intradermal route is adequate for the administration of a certain agent in one animal, that this route will also be efficacious in another animal, or in man for a variety of related or other agents.

What one *can* hope to do with some profit is to ask the question in a much larger context and in much broader terms: Is this category of agents effective, in a variety of test species, only by systemic application, or is it also effective by a variety of topical or local administrations? In other words, there must be a logical and ordered flow from the general to the particular, by which one also hedges one's bets. Thus, in this example, even if one will not obtain definitive information about intradermal injection in man, one may be left at least with a generality: Some sort of topical or local treatment is or is not likely to give the same results as systemic administration. A framework of *general applicability* to the clinical situation can thus be established, and more precise information must then be sought from human trial.

To summarize this point, then, I would say that breadth of the comparative animal experimentation system is a primary requisite in the design of models that are to have some implication for man. An overlapping of sets of test parameters in different species is necessary to gather some perspective, and attention must clearly be focused on the intrinsic nature of the biologic entities which are the models and the objects of investigation.

It may be of little significance—to take another obvious example—to study experimentally induced bone or lung metastases in a model animal-tumor relationship if such metastases do not occur naturally with analogous neoplasms in that species, or in man.

Clearly, our attempts at comparison must always be oriented with a view to the boundaries between what resembles nature and what is wholly artifactual.

Although it is necessary in practically any investigation to focus on 1 or 2 parameters at a time, we should do so with a clear awareness that individual parameters must always be seen as facets of a much larger, encompassing situation in nature if we are to have any hope of finding pertinency of animal models to the human condition.

With this brief introduction, I would like to set the framework for our discussions this afternoon. Our aim is to evaluate the present state, and the most profitable future directions, of investigations in animal models designed to have immediate implication for immunotherapy of cancer in man.

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Histologic and Ultrastructural Studies of Tumor Regression in Inbred Guinea Pigs after Intralesional Injection of *Mycobacterium bovis* (BCG)[1,2]

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SUMMARY—A comparative histopathologic study was performed on inbred guinea pigs at the site of a transplanted syngeneic hepatocarcinoma and in the draining lymph nodes, in the presence and absence of *Mycobacterium bovis* strain BCG. BCG was injected into the growing intradermal tumor 7 days after transplantation, when the tumor had metastasized to the first regional lymph node. The histopathology was compared with that of saline-inoculated tumors and with that of animals from which tumors had been surgically excised 7 days after transplantation. In this system, guinea pigs die 60–90 days after intradermal injection of 10^6 hepatocarcinoma cells in the absence of BCG treatment. The results demonstrate that intradermal tumors completely regress after treatment with BCG and that regional lymph-node metastases are eliminated. The mechanism is a BCG-mediated granulomatous reaction at both the tumor site and the regional lymph node. As detected both histologically and ultrastructurally, histiocytes appear to be the major effector cells in this reaction. In this syngeneic tumor system, a conventional lymphoproliferative response of the regional node, in the absence of histiocytosis, is clearly insufficient to inhibit tumor growth. The cytopathic effect of the activated histiocytes on tumor cells is mediated by cell-surface contact; we observed sizable areas of apparent fusion of the cellular membranes of histiocytes and hepatocarcinoma cells. Thus destruction of target cells by nonphagocytic mechanisms may be a phenomenon related to activated but not specifically sensitized cells of the macrophage-histiocyte series.—Natl Cancer Inst Monogr 39: 71-84, 1973.

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

FOR A NUMBER OF YEARS it has been shown that administration of *Mycobacterium bovis* strain BCG suppresses tumor induction or alters the rate of tumor growth in several mouse and rat experimental models (*1–6*). Only recently, however, has an adequate experimental model been developed that fulfills many of the requirements for a study of effective immunotherapy of established tumors that have metastasized (*7, 8*). This model consists of a transplantable syngeneic hepatocarcinoma in inbred guinea pigs. With this experimental system, it has been demonstrated that in most inbred guinea pigs, palpable intradermal tumors and detectable metastases in regional lymph nodes can be eliminated by injection of an adequate amount of viable BCG. Untreated guinea pigs die as a consequence of metastases to vital organs 60–90 days after intradermal injection of 10^6 syngeneic hepatocarcinoma cells, and somewhat later when surgical excision of the skin tumor is performed in place of the BCG therapy.

Our efforts have been devoted to the histopathology of tumor regression and elimination of regional metastases both at the light and electron microscopic levels (*9–11*). The objectives of our study have been to determine the characteristics and immunologic requirements of the tuberculous lesions in the tumor site and regional lymph nodes, as well as to define the primary cytotoxic lymphoid cell and the mechanism by which it destroys tumor cells.

PROCEDURE AND RESULTS

We evaluated the histopathology of a transplanted syngeneic hepatocarcinoma at the intradermal site (upper right quadrant) and in the draining lymph node in the presence and absence of BCG (Phipps strain TMC 1029). Living BCG (24×10^6) was injected into the intradermal tumor 7 days after transplantation, a time established by histologic examination to be the earliest stage of metastasis to the draining lymph node. The histopathology was compared to that of saline-inoculated tumors and to that of animals from which tumors had been excised at day 7. Normal guinea pigs receiving a single intradermal BCG injection were also evaluated as controls.

It is important to qualify the terminology to be associated with these descriptions, because of the difficulty in interpreting the extensive literature on classic tuberculin granulomatous disease. The general term "histiocyte" is used to refer to a cell type that is distinguishable from active macrophages or phagocytic mononuclear cells and consistent with the characteristics of the epithelioid cells associated with granulomatous reactions (*12*). It is accepted that these may be different forms of the same cell type (*13*). Also, it is recognized that, in the early host response, phagocytosis of a limited number of bacilli by macrophages initially activates the cell compartment, as described by Dannenberg (*14*). However, the general absence of active phagocytosis or representative numbers of cell inclusion bodies in the enlarged BCG-activated compartment of responding cells tends to argue against phagocytosis as their sole or primary function. We reject the terms "epithelioid" and "epithelial-like cells" because they are based on a superficial resemblance and are misleading with respect to the origin and function of the cells.

One day after saline injection, a slight mononuclear cell infiltrate was at the border of the subcutaneous tumor. This infiltration consisted mainly of lymphocytes and occasional histiocytes. At this time, the regional lymph node, the superficial distal axillary (SDA) node, did not differ from that observed before saline injection (i.e., occasional tumor cells in the subcapsular marginal sinus and no marked alterations in the cortical and paracortical regions). Metastasis increased in these nodes 8 days after saline injection, and an apparent hypocellularity of the paracortical region was observed, with negligible germinal center activity. Twenty-five days after saline injection, the greatly enlarged SDA lymph nodes were entirely metastatic, containing only small remnants of the lymphoid components (fig. 1). The remaining animals in this group began to die as a consequence of metastases 60 days after tumor implantations.

Twenty-four hours after tumor excision, no marked alterations were detected in the SDA lymph nodes, although metastatic tumor cells were observed in the subcapsular marginal sinus of these nodes. Our impression was that there were fewer tumor cells than had been observed

24 hours after the injection of saline into the tumor. At days 1 and 4, there was considerable germinal center and immunoblast activity in the lymph node cortex. By 8 days after tumor excision, metastases were extensive in the SDA node and had penetrated the cortex and the medullary sinuses. At 25 days, there was metastatic growth of tumor cells in both the cortex and medullary sinuses of the node, though it was much less than in the saline-inoculated groups. A marked hyperplastic germinal center reaction in the cortical area was a unique feature of the SDA lymph nodes after excision of the tumor (fig. 2) This was a prominent alteration of the cortex, implying a unique reaction to metastatic tumor cells not seen in the presence of a growing tumor as in the saline-inoculated group. It is apparent from the extensive metastases that tumor cells grow well despite such a lymphoproliferative response. Animals died as a consequence of metastasis at approximately 90 days after tumor implantation.

Twenty-four hours after BCG injection, an acute inflammatory reaction was observed in the tumor site. This was characterized by edema and both perivascular and intervascular infiltration of polymorphonuclear and mononuclear cells. The lymphoid components of the cortical and paracortical regions of the SDA lymph nodes were not altered at this time. Metastasis of the tumor to the subcapsular marginal sinus of the SDA nodes was more extensive 1 day after BCG than in saline-inoculated or tumor-excision guinea pigs. We assumed that there was a greater influx of tumor cells or tumor emboli via the afferant lymphatics as a result of the acute inflammatory response and the vascular alterations at the tumor site. In acid-fast stained preparations, mycobacteria were detected in the marginal sinus; the organisms were associated with phagocytic reticular cells but were never in direct contact with tumor cells.

At 4 days after BCG injection, the architecture of the SDA lymph node was markedly altered (fig. 3). Penetration of tumor cells into the cortex had destroyed its follicular structure, and active germinal centers were absent. Noncaseous granulomas were present throughout the cortex, bordering the rim of metastatic tumor cells in the node. A few mycobacteria were occasionally observed in these granulomas.

Cytologically these focal granulomas consisted of concentrations of histiocytes, and mitotic figures were commonly present (fig. 4). Many lymphocytes were present in the medullary sinuses, as well as a large proportion of histiocytes, a feature unique to the animals treated with BCG. The nuclei of these histiocytes were similar to those of cells constituting the granulomas of the cortex. Frequently the histiocytes were closely associated with the tumor cells, and some of the tumor cells showed definite signs of degeneration.

Eight days after BCG injection (15 days after tumor cell injection), the architecture of the ipsilateral SDA lymph nodes consisted of coalesced granulomas occupying the major portion of the cortical and paracortical regions of the node (fig. 5). This pattern was reminiscent of a syncytial histiocytosis. Tumor cells within these reaction centers showed signs of degeneration and were relatively sparse.

At 25 days after intralesional injection of BCG, the ipsilateral SDA nodes were smaller than at the previous intervals, and the BCG histiocytosis occupied most of the node. Distinct sinuses were rare, and areas of focal necrosis were present throughout the node. Small but discrete regions of lymphoid components could be observed (fig. 6). No tumor cells or mycobacteria could be detected in the regional nodes at this period. The intradermal transplantation site was generally fibrotic, with some evidence of BCG-induced histiocytosis. No intact tumor cells could be distinguished, but there were numerous areas of focal necrosis.

Since the level of resolution of these histologic studies prevented elucidation of the cytologic characteristics of the interaction between histiocytes and tumor cells, ultrastructural studies were performed. The granulomas studied in SDA lymph node 4 days after BCG treatment consisted of aggregations of histiocytes which were morphologically indistinguishable from histiocytes observed in the tumor or from activated stromal reticular cells in the lymph node. Histiocytes composing the granulomas were characterized by extensive interdigitations of cytoplasmic processes with adjacent histiocytes. These interdigitations produced a very dense

interlocking of cells to form the basis of what has been incorrectly termed "syncytial histiocytosis" by light microscopists.

Numerous examples of interactions between histiocytes and tumor cells were observed at the tumor site and in the various regions of the draining lymph nodes between 4 and 16 days after intralesional injection of BCG. Often observed was degeneration of tumor cells that were associated with or surrounded by histiocytes. Figure 7 illustrates histiocytes and their interactions with tumor cells in the medullary sinuses of SDA lymph nodes draining the sites of tumors injected with BCG. Cytologically there was considerable similarity in appearance between these histiocytes and those seen in the granulomas, the subcapsular marginal sinus, and the tumor site. Their cytoplasm contained variously sized vesicles of rough-surfaced endoplasmic reticulum, which was sometimes well developed. Intracellular microfilaments were occasionally present but intercellular desmosomes were not. Because of their large nuclei and prominent nucleoli, these cells have been called epithelioid cells by other investigators. Although kinetic evidence concerning the specific origins of these cells is not yet available, their morphologic characteristics suggested a mesenchymal origin from stromal or primitive reticular cells.

Intradermally injected hepatocarcinoma cells proliferated as nests of cells dispersed among connective tissue elements. Histiocytes and hepatocarcinoma cells frequently interacted in the tumor site. Destruction of tumor cells by histiocytes morphologically similar to the activated histiocytes described in lymph nodes is illustrated in figure 8. Degenerating tumor cells became dense and irregular in shape and had pyknotic nuclei (figs. 8*a, b*). On the ultrastructural level (fig. 8*c*), histiocytes were in direct contact with the surfaces of degenerate hepatocarcinoma cells. Areas of apparent fusion of the plasmalemmae of histiocytes and hepatocarcinoma cells could be seen. While we accept that morphology alone cannot determine the functional mechanism of cell interactions, figure 9 suggests areas of apparent fusion of the membranes of histiocytes and tumor cells. These areas were found with considerable regularity in the regional lymph nodes (figs. 9*a, b*) and seemed to be an integral part of the reaction of BCG-activated histiocytes against the hepatocarcinoma cells. Similar areas of cellular contact were observed at the tumor site, some being relatively long and others alternating with short areas of detachment (figs. 9*c, d*). These may have represented biased sections of the cellular plasmalemmae, but at the very least there was an intimate association of histiocyte surfaces with surfaces of tumor cells, many of which showed signs of degeneration.

CONCLUSION

Our studies demonstrated that, when guinea pigs bearing intradermally transplanted syngeneic tumors were inoculated intralesionally with BCG, an immediate inflammatory response at the tumor site was followed by a typical BCG-mediated granulomatous reaction in the lesion, as well as in the regional lymph nodes. This reaction contributed to the complete elimination of the tumor and total elimination of metastases in the regional lymph nodes. From comparison of BCG-treated normal and tumor-bearing guinea pigs, the qualitative and quantitative aspects of the granulomatous reaction appeared to be independent of the tumor and were solely a function of the specific immunologic reaction provoked by the microorganism. Another direct correlation between the BCG-mediated granulomatous responses in normal and tumor-bearing animals was the requirement of a BCG-specific delayed-hypersensitivity reaction in the therapy model (*15*). Thus, in a tuberculous lesion produced within an established tumor, the changes attributed to the ability of activated histiocytes to destroy the tubercular organisms could also be associated with their ability to destroy the tumor nonspecifically.

A major contribution of this study was the finding that the histiocytes in various reactive regions—infiltrating at the tumor site, migrating from the tumor site to the subcapsular marginal sinus of the lymph node via the afferent lymphatics, autochthonous to the lymph node, and infiltrating into medullary sinuses—were morphologically indistinguishable on an ultrastructural level. This led us to believe that these were all forms of the same class of cells. We

propose that infiltrating mononuclear cells enter as free histiocytes, carry on an activated sequence of events associated with mycobacterial destruction, and eventually form granulomas in conjunction with the proliferative activity of stromal reticular cells. As the histiocytosis proceeds, there is differentiation with phagocytosis and the formation of reticular fibers. The reticular fibers form delicate interlacing networks, and ultimately a diffuse histiocytosis is established from the coalescence of these granulomas.

It is important that we consider the mechanism of BCG-mediated tumor regression and elimination of regional lymph node metastases as a nonspecific reaction mediated by the physiologically altered and possibly detrimental environment created by the granulomatous reaction at the tumor site and in the draining lymph node. While this may account for part of the destruction of the metastatic growth, it still does not completely explain the subtle interactions between histiocytes and tumor cells in the lymph node sinuses, where there are obvious signs of tumor cell degeneration. Ultrastructural evidence indicates that there are sizable areas of apparent fusion of the cellular membranes of histiocytes and hepatocarcinoma cells within the regional lymph nodes and the transplantation site, lending support to the idea that the cytopathic effect of the histiocytes may involve a cell-surface contact phenomenon. This response is reminiscent of the interactions between "activated" histiocytes and tumor cells described by others in in vitro as well as in vivo systems (*16–18*). The cytopathic mechanisms by which such "activated" histiocytes destroy tumor cells are not completely understood. Recent evidence, however, indicates that target cell destruction by a nonphagocytic mechanism might be a phenomenon related to activated, but not specifically sensitized, cells of the macrophage-histiocyte compartment (*19*).

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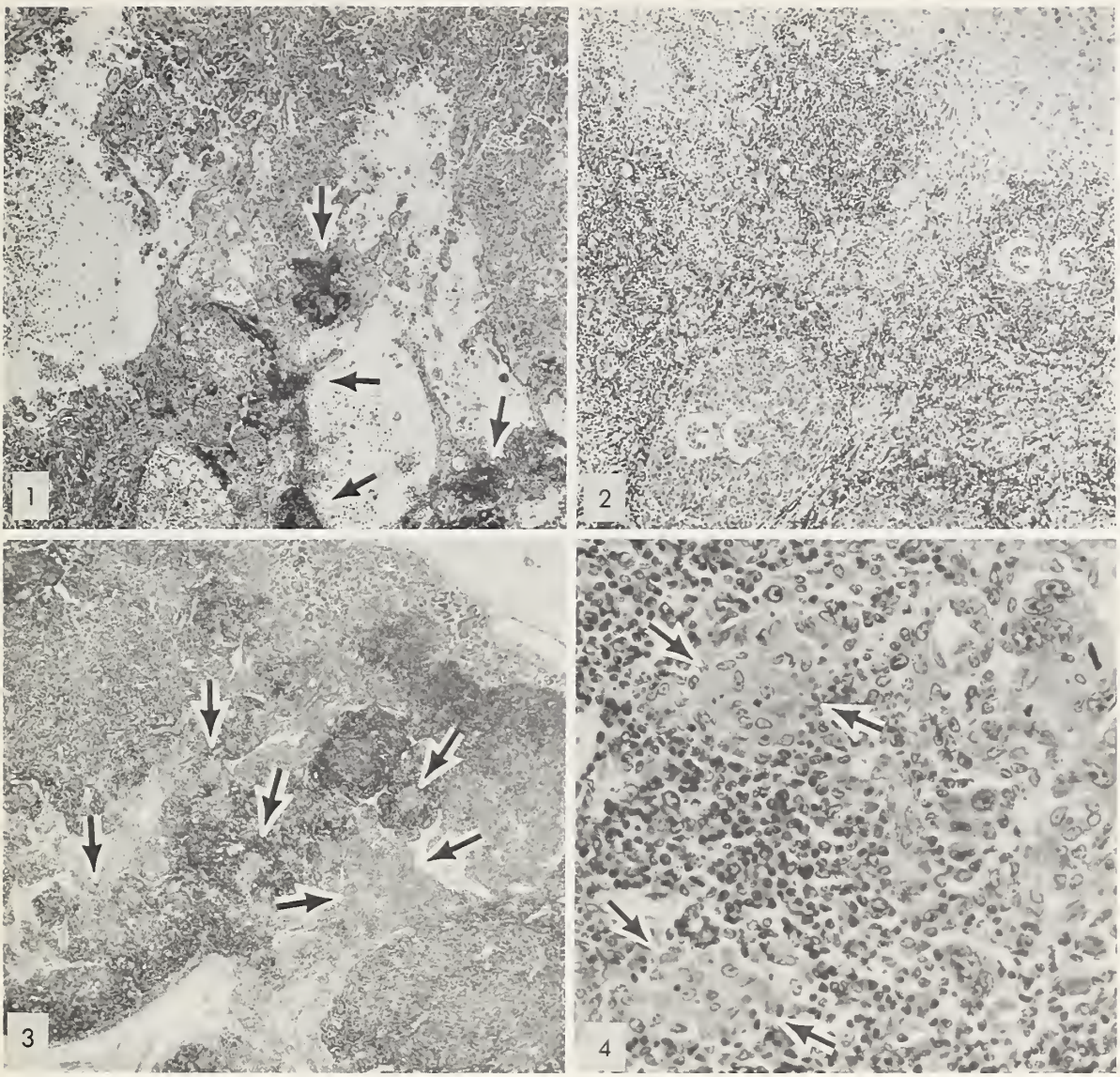
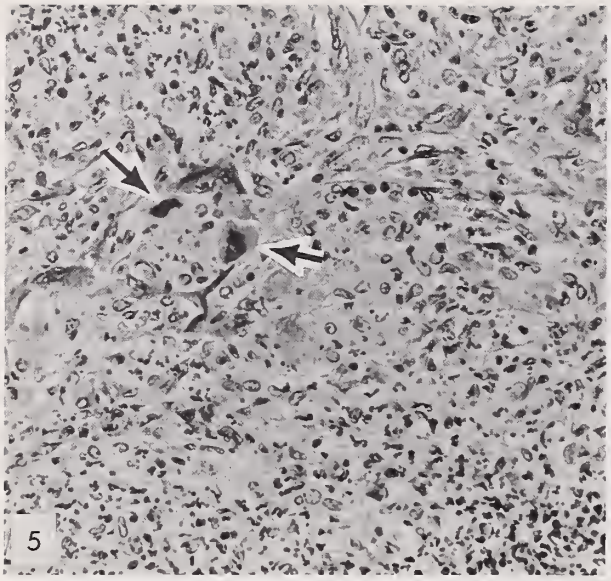


FIGURE 1.—Metastatic SDA lymph node 25 days after intratumor saline injection. *Note* residual lymphatic tissue (*arrows*) and cystic condition of node. × 30

FIGURE 2.—SDA lymph node, 25 days after tumor excision. *Note* hyperplastic germinal centers (GC) and extensive metastasis (*upper right*). × 125

FIGURE 3.—SDA lymph node draining BCG-inoculated tumor, 4 days after BCG injection. *Note* extensive metastasis (*upper right*), histiocyte-infiltrated medullary sinus (*lower left*), and granulomas (*arrows*). × 30

FIGURE 4.—SDA lymph node of BCG-inoculated tumor-bearing guinea pig, 4 days after BCG injection. *Note* tumor cell growth into cortex (*upper right*) and discrete histiocytic granulomas (*arrows*). × 300



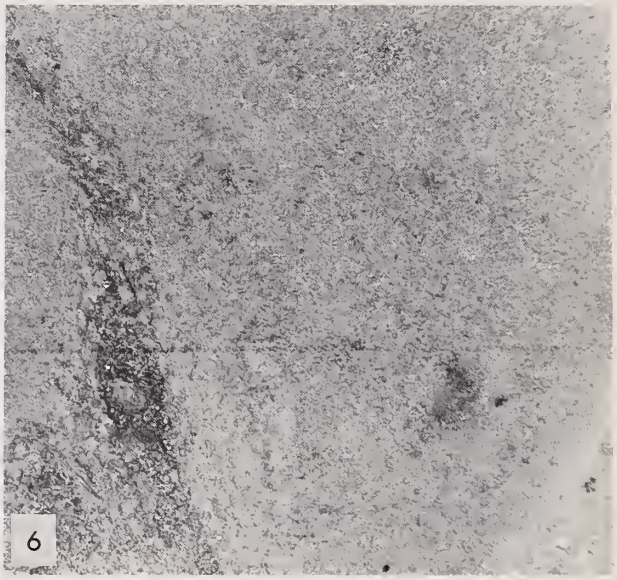


FIGURE 5.—Degenerating tumor cells (*arrows*) trapped in granulomatous reaction centers of SDA lymph nodes, 8 days after intratumor BCG injection. × 300

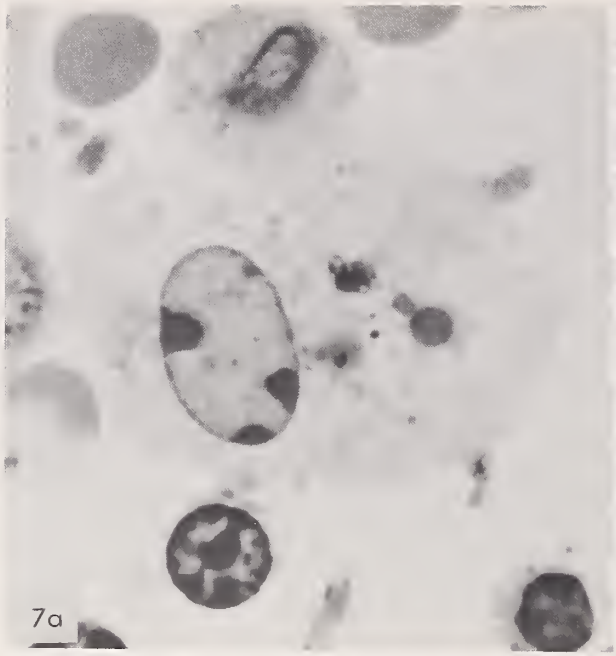
FIGURE 6.—BCG histiocytosis of SDA lymph node, 25 days after intratumor BCG injection. *Note* residual lymphoid component (*lower left*) and focal necrotic mass (*lower right*). × 30

FIGURE 7.—Histiocytes and metastatic tumor cells in medullary sinuses of SDA lymph node after BCG treatment.

a) *Note* multiple nucleoli and dense bodies in cytoplasm, 4 days after BCG. × 1700

b) Histiocytes in contact with 2 metastatic tumor cells, 8 days after BCG. × 640

c) Multiple nucleoli of histiocytes have a prominent fibrillar nucleolonema. A moderate amount of rough-surfaced endoplasmic reticulum and free polyribosomes typify the cytoplasm. Sinus wall is to the *right* of this field, 4 days after BCG. × 7500



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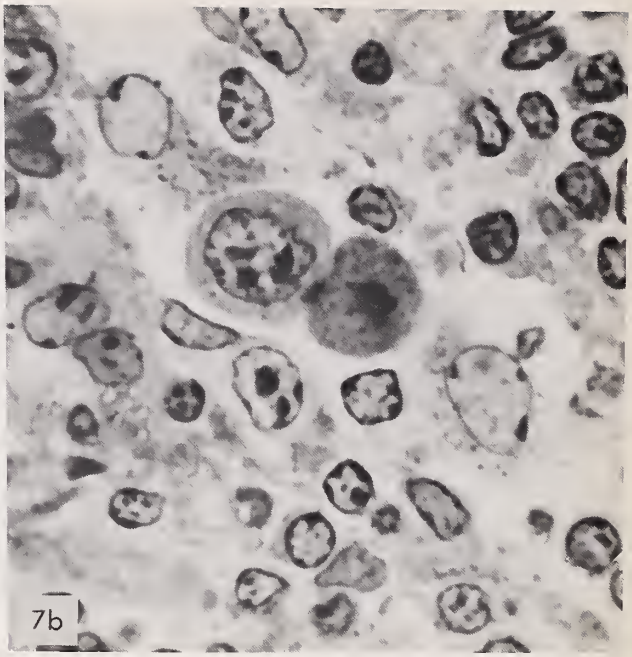

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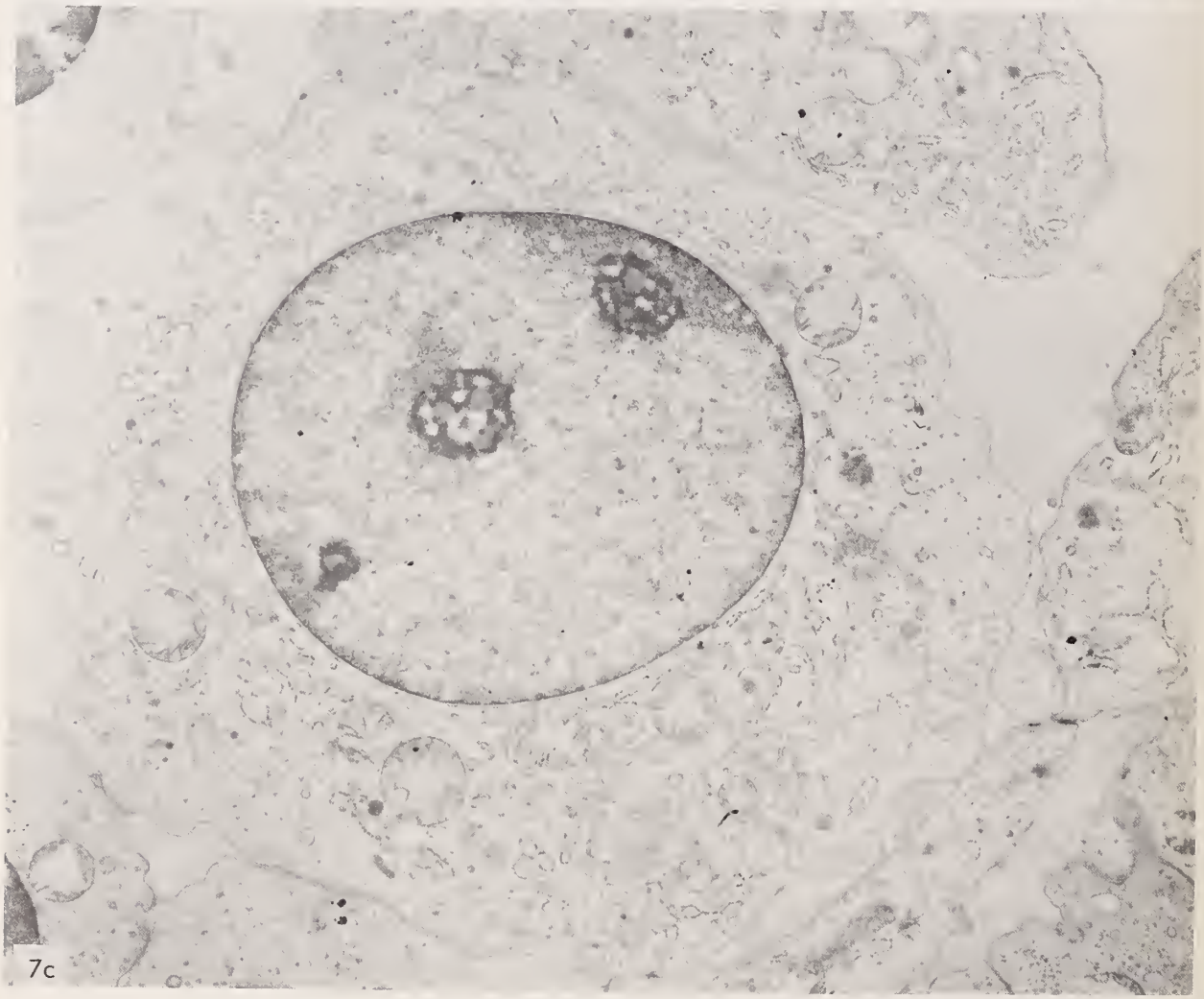

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FIGURE 8.—Tumor cell destruction at subcutaneous tumor site.

a) Histiocytes (*arrows*) among hepatocarcinoma cells. Both mitotic and degenerate tumor cells are present, 8 days after BCG. × 640

b) Histiocytes (*arrows*) in contact with many degenerate, pyknotic hepatocarcinoma cells, 16 days after BCG. × 570

c) Areas of apparent fusion (*arrows*) of plasamalemmae of histiocytes (H) and a dark hepatocarcinoma cell (T) are present. *Note* also numerous intracytoplasmic channels in histiocytes that contain electron-dense material, 8 days after BCG. × 7500

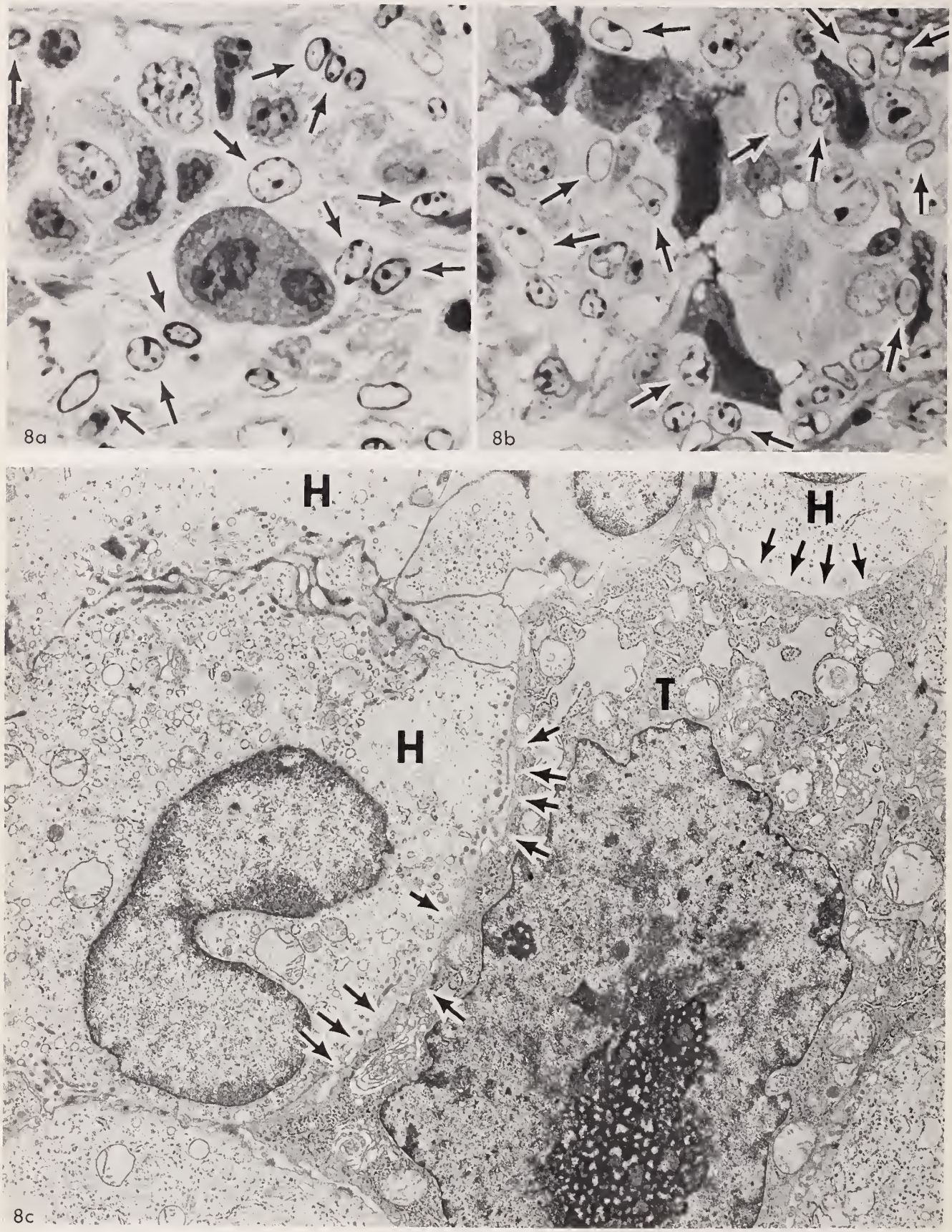
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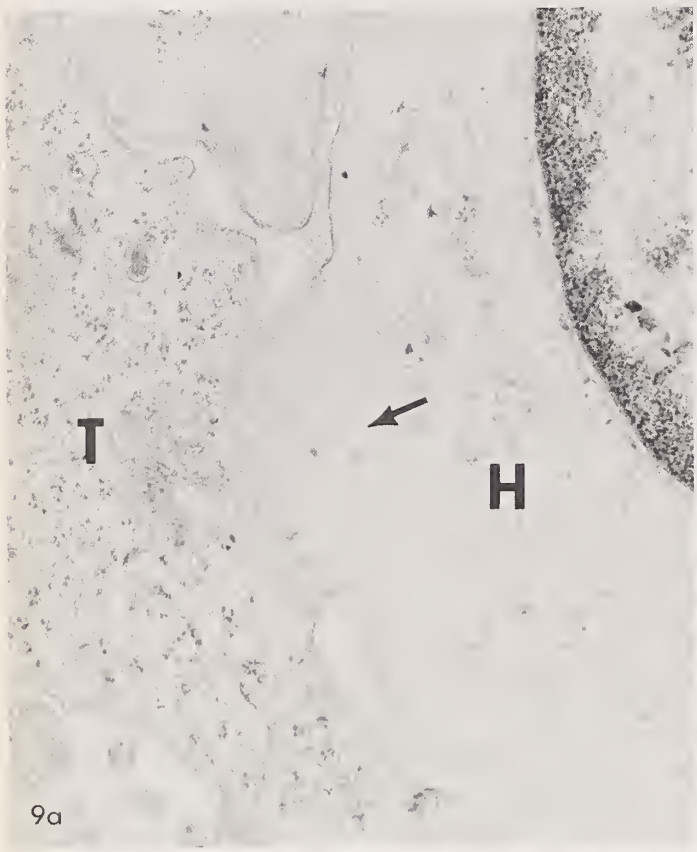
FIGURE 9.—Areas of apparent adhesion (*arrows*) between plasmalemmae of histiocytes (H) and hepatocarcinoma cells (T).

a, b) Subcapsular sinus of SDA lymph node, 8 days after BCG. × 16,750; × 28,000

c, d) Subcutaneous tumor transplantation site, 8 and 16 after BCG. × 20,000; × 24,000



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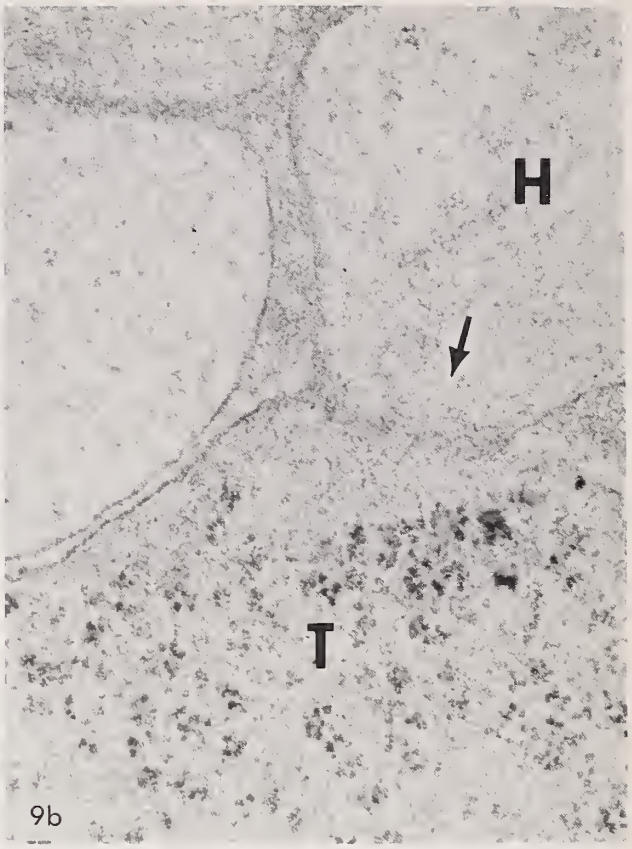

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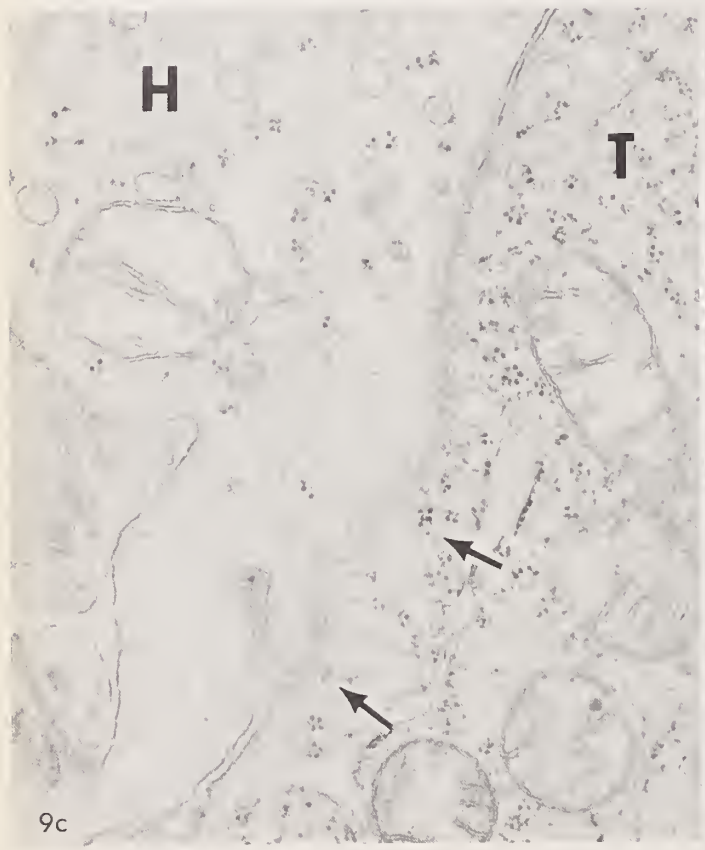

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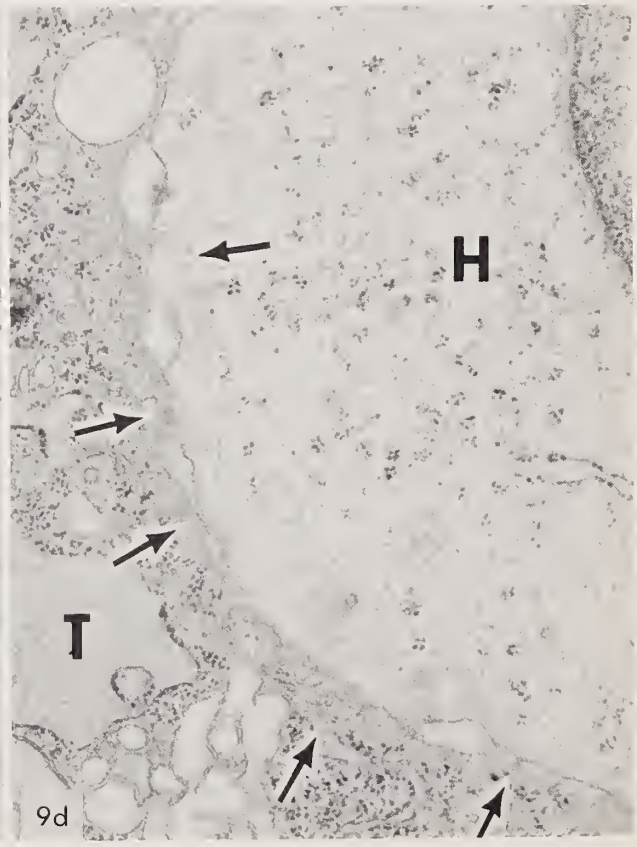

9d

DISCUSSION

D. W. Weiss: As this is the only formal presentation scheduled this afternoon dealing with histology, we should spend a few minutes discussing aspects of this subject.

M. Chase: Was the tumor regressing at the time that the histiocyte tumor cell interaction was observed?

M. G. Hanna, Jr.: Yes.

Taylor: Some of your photographs are very reminiscent of the photographs that were published by Porter (*British Journal of Medicine*) dealing with the first findings in the rejection of kidney transplant. Porter showed the histiocyte from the host reacting with the endothelial cells in the hairy tubular capillaries, which is identical to what you've shown here.

Hanna: We are very careful to point out that there is always a possibility that on the ultrastructural level evidence of cell fusion might be a result of biased sectioning. However, the numbers of points of fusion were very frequently along a length of cell membrane and were so often seen that we find it extremely difficult just to eliminate them as a result of biased sectioning. They definitely indicate that there is a buffeting of the tumor cell surface by the histiocytes and that there is an intimate association and contact of the tumor cell–histiocyte cell surfaces. Whether there is indeed continuity of the cytoplasm between the 2 cells would have to be demonstrated in future studies.

Taylor: There are 2 workers in Montreal who are interested in metastatic tumor in the liver, and they see the same sort of reaction. The reaction is not immunologic; it's just the growth of the metastatic tumor cell involving the liver cell. Again you see this contact of cell to cell.

Hanna: We also observed association between tumor cell surfaces; however, these are classic desmosome structures, a recognized phenomenon of intercellular contact. The association between tumor cell and histiocytes, however, does not have the ultrastructural morphology of desmosomes. This kind of association and its relationship to cell destruction are unique from what I think you are describing and what we have described as the tumor cell-tumor cell interaction.

Weiss: Are there any other questions on this area of histology?

Let us proceed, then. We will now have 3 short presentations on the general topics of technical aspects pertaining to route, dose, and number of injections.

BCG Immunization for the Lymphoid Leukemia Line (LSTRA) in CDF_1 Mice[1]

Sotiros D. Chaparas, *Ph. D.*,[2] Michael Chirigos, *Ph. D.*, Jack Pearson, *Ph. D.*, *and* Neal Sher, *M.D.*,[2] *Mycobacterial and Fungal Antigen Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, and Viral Biology Branch, National Cancer Institute,*[3] *Bethesda, Maryland 20014*

SUMMARY—The importance of route, dose, and timing of BCG administration was studied in leukemic mice in remission after drug therapy.—Natl Cancer Inst Monogr 39: 87–88, 1973.

IN AN AREA as complex as we have been discussing, several factors make it clear that we are dealing with a state of *balance*. Factors determining this balance include:

1) Cellular immunity versus blocking antibody
2) Immune responsiveness versus tolerance
3) Specific antigen availability in syngeneism
4) Antigenic competition
5) Reticuloendothelial system deficiency

Whenever we have a delicate balance playing a role in immune recovery from cancer, a rational approach to treatment and immunotherapy demands definition and quantitation. Many of the clinical and experimental studies reported in the literature suffer from a lack of definition and quantitation. It is important to know what strain of BCG or other agent is used, how many viable units are used, how these values are determined, how the strain is grown, what the route of administration is, etc. It is also important to develop laboratory tests to assess the effectiveness of vaccines.

To illustrate the importance of route, dose, and timing of BCG administration, the following experiments were performed in CDF_1 mice which had been given subcutaneous (sc) doses of 1×10^4 tumor cells. On day 7, the disease was systemic; the animals were put into remission with 1,3-bis (2-chloroethyl) -1-nitrosourea (BCNU). At various intervals, mice were given variable doses of the Phipps strain of BCG.

In table 1 we have determined the effects of dose of BCG administered by the intradermal (id) route on days 10 and 16 on immune survival after treatment with BCNU. Clearly, BCG administration on day 10 (3 days post drug administration) was effective over at least a 4-log range. The mice died early from BCG disease at the highest dose of 8×10^8 organisms. Doses from 8×10^2 to 8×10^6, equally effective, induced about 50–70% survivals. However, if the same doses were given on day 16, a dose response could be observed and the lowest dose of 800 organisms elicited only a 10% survival. Higher doses were more effective but not as effective as those similar doses given to groups at day 10. The id route thus is effective for immunotherapy over at least a 4-log dose route. This route would not be satisfactory for comparisons of the efficacy of strains of BCG vaccine in protection.

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[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Food and Drug Administration.

[3] National Institutes of Health, Public Health Services, U.S. Department of Health, Education, and Welfare.

TABLE 1.—Effect of dose and time of administration of BCG against LSTRA following drug therapy

Day of BCG administration	Route	Dose of BCG	Percent survivors
10	id	8×10^2	70
		8×10^4	50
		8×10^6	60
		8×10^8	33
16	id	8×10^2	10
		8×10^4	30
		8×10^6	40
		8×10^8	40
Drug alone	—	—	20
Control	—	—	0

TABLE 2.—Effect of route and dose of BCG against LSTRA following drug therapy

Day of BCG administration	Route	Dose of BCG	Percent survivors
10	id	8×10^2	75
10	id	8×10^6	73
13	id	8×10^2	83
13	id	8×10^6	90
10	ip	8×10^2	75
10	ip	8×10^6	66
13	ip	8×10^2	75
13	ip	8×10^6	66
10	ia	8×10^2	100
10	ia	8×10^6	58
13	ia	8×10^2	83
13	ia	8×10^6	58
10	fp	8×10^2	50
10	fp	8×10^6	58
13	fp	8×10^2	33
13	fp	8×10^6	92
Drug alone	—	—	27
Control	—	—	0

* ia = inguinal and axillary regions.
† fp = footpad.

In table 2, results of the effects of route of administration of 2 doses of BCG given on days 10 and 13 are explored. The id and intraperitoneal (ip) routes were both effective, giving high survival over a 4-log dose of viable BCG. Administration of the BCG organisms sc in the inguinal and axillary regions was most effective at day 10 and at the lowest dose of 800 organisms. When 8 million organisms were instilled, the degree of protection was lowered. Whether some blocking antibody is produced or some other factors are involved is unknown. We are studying this point.

The footpad route of BCG administration at day 13 seemed to be effective and permitted a quantitation in the positive direction. The smallest dose was not as effective as the larger dose. More points are needed to establish a dose-response curve, but this route may permit comparison of strains for efficacy.

These results indicate a balance required for only a single tumor in a single species. We are also trying to determine criteria for optimal immunization toward other tumor systems. We hope that this type of quantitative approach may provide us with animal models to study the intricacies of BCG immunotherapy, the evaluation of BCG strain differences, and a more rational approach to immunotherapy.

Immunoprophylaxis and Treatment of Leukemia in AKR Mice: Repetitive Use of BCG[1]

Michael R. Mardiney, Jr., Peter C. Ungaro, and Walter P. Drake, *Section of Immunology and Cell Biology, National Cancer Institute,[2] Baltimore Cancer Research Center, Baltimore, Maryland 21211*

SUMMARY—Leukemic AKR mice, randomized at 8 weeks of age, were treated every 2d week up to 42 weeks of age with intraperitoneal injections of varying doses of BCG or saline. Despite increased longevity compared to the saline controls, all the BCG-treated animals eventually developed tumors.—Natl Cancer Inst Monogr 39: 89–90, 1973.

I SHALL present 2 tables relating to the question of the effect of repetitive administration of BCG in the immunoprophylaxis of spontaneous leukemia in AKR mice. In this model, AKR mice were randomized at 8 weeks of age and treated every 2d week up to 42 weeks of age with intraperitoneal injections of BCG or saline.

The BCG was obtained from the Institute of Tuberculosis Research, Tice Laboratory, Chicago, Illinois. All BCG was from a single lot, each vial containing 1.81×10^8 viable organisms.

Table 1 presents the effect of BCG on the longevity of AKR mice. The concentrations of viable organisms injected into each of 6 different groups of AKR mice varied from 5.4×10^6 to 0.17×10^6. At week 21, the number of survivors was markedly decreased in that group receiving the highest dose of BCG (5.4×10^6). Gross examination of autopsied animals within this group revealed no leukemia. Histologic examination, however, revealed the presence of granulomas in liver, spleen, and lungs along with the presence of microscopic leukemia. Although it was difficult to pinpoint the exact cause of death in these mice, our assumption was that both BCG and leukemia initiated a combined adverse effect that resulted in the animals' deaths. However, all other BCG-treated groups had a greater increase in survival than their saline-treated counterpart. When these BCG-treated animals died, however, autopsy results indicated that all animals had some tumor. In most autopsied mice, the presence of tumor was easily identified by gross examination. In others, histologic examination was needed to establish the presence of leukemia.

Table 2 is a χ^2 probability analysis of the data in table 1. The number of animals surviving in all BCG-treated groups, excluding the group receiving the highest dose of BCG, was averaged for these determinations. A marked statistical difference in survival existed between the BCG-treated animals and the saline-treated controls from 36 weeks of age to 48 weeks of age.

We believe the present work is additional evi-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

TABLE 1.—Longevity of AKR mice receiving BCG*

Cage No.	Number of organisms	Age of mice (wk)														
		8	13	16	19	21	27	29	31	34	36	38	40	44	46	48
		Mice surviving/group														
1	5.40×10^6	20	20	19	14	9	9	7	7	7	6	5	5	5	5	4
2	2.70×10^6	20	20	19	18	16	15	15	14	13	11	9	6	4	4	4
3	1.35×10^6	20	19	19	19	19	19	15	13	11	10	8	5	4	3	3
4	0.68×10^6	20	19	19	18	18	16	16	16	14	14	13	12	10	8	7
5	0.34×10^6	20	20	20	20	20	20	18	16	11	8	7	6	5	4	2
6	0.17×10^6	20	20	20	20	20	20	18	17	13	11	9	8	5	5	5
7	Saline	20	20	18	18	17	17	13	8	7	5	3	2	1	1	1

* 8-week-old AKR mice were randomized into groups of 20 and treated with the indicated number of viable BCG organisms or saline every 2 weeks. The numbers of surviving animals were determined at the indicated times. The last dose of BCG was given to mice at 42 weeks of age.

TABLE 2.—Probability analysis by χ^2 of data in table 1

Age (wk)	Mice surviving*		P value
	Groups 2–6	Group 7	
27	18.0	17	<0.99
29	16.4	13	< .50
31	15.0	8	< .02
34	12.4	7	< .05
36	10.8	5	< .01
38	9.2	3	< .001
40	7.4	2	< .001
44	5.6	1	< .001
46	4.8	1	< .001
48	4.2	1	< .01

* The number of animals surviving in cages 2–6 was averaged for these determinations.

dence indicating that adjuvant immunotherapy may have a positive effect on a host predestined to develop tumor as well as on a host bearing tumor. The AKR-BCG model presented is of interest because, despite increased longevity, all mice developed tumor. The question is whether different regimens or different adjuvants may improve on these results.

BCG and the Lympho-Reticuloendothelial System[1]

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SUMMARY—It is generally agreed that a stimulated and active immune system is operative in the suppression of neoplastic cells. BCG is one of the most active stimulators of the immune system as noted by the response of the lympho-reticuloendothelial system (lympho-RES). The route of administration, the species of animal, and the dose are important factors that determine the degree of stimulation. In the guinea pig and in man the dermal route of administration of BCG is effective but to a lesser degree than the intravenous (iv) or aerosol (AV) routes. The dose of BCG for a specific immunizing effect can be small, but to obtain a nonspecific effect relatively large doses are needed ($\geq$ 1 mg). The specific effect may last for years and can be made operative quickly by a specific antigen. The nonspecific effect is of short duration and cannot be stimulated by nonspecific agents such as neoplastic cells. Therefore, large doses of specific agent must be present constantly to maintain the nonspecific effect. In the guinea pig model, relatively large doses of BCG by the iv, AV, intradermal, or oral routes stimulated the lympho-RES, the stimulation being most effective by the iv and AV routes and least effective by the oral route. Specific stimulation at periods up to 14 months generally resulted in a marked reactivity of the RES.—Natl Cancer Inst Monogr 39: 91–104, 1973.

IT IS generally agreed that a stimulated and active immune system is operative in suppressing neoplastic cells. BCG is one of the most active stimulators of the immune system as noted by the response of the lympho-reticuloendothelial system (lympho-RES) (*1, 2*). Its specific (*3*) and nonspecific (*2*) activity is greatly enhanced with viable organisms. The route of administration, the species of animal, and the dose are important factors that determine the degree of stimulation. In some species of animals (e.g., mouse, monkey) the dermal route of administration of BCG is relatively ineffective; whereas the intravenous (iv) or aerosol (AV) routes are highly effective (*4*). In the guinea pig and in man, the dermal route is effective but to a lesser degree than the iv or AV routes (*5, 6*).

The dose of BCG for a specific immunizing effect can be small (10^{-6} mg in the mouse, 5–20 organisms in the guinea pig); but to obtain a

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

nonspecific effect as against sarcoma 180, relatively large doses are needed (1 mg) (7). The specific effect may last for years and can be made operative very quickly by being stimulated by a specific antigen; on the other hand, the nonspecific effect is only of short duration and cannot be stimulated by nonspecific agents, e.g., neoplastic cells. Therefore, large doses of the specific antigen must be present constantly to achieve and to maintain the nonspecific effect (2).

MATERIALS AND METHODS

Guinea pigs 300–400 g (suckling pigs 2–7 days old for the oral route) were inoculated by the iv route (through the right ventricle—10–15 mg), by AV (in chamber—3,000 clones), by the intracutaneous route (10 mg), and by the oral route (5 mg doses daily for 3 days—15 mg). They were killed at regular intervals (10 min to 14 months). The lungs were fixed in situ with Zenker's solution before the chest was opened. Histologic examinations were made of the lungs, lymph nodes, liver, spleen, and kidneys; for the animals given oral injections, the intestines were also examined. The tissues were run through paraffin and stained with hematoxylin and eosin and carbolfuchsin—methylene blue.

Control animals of varying ages (2 days to +0 to 1½ yr) were studied histologically as above. Concomitant blood counts, differentials, and tuberculin testing at specific intervals were done.

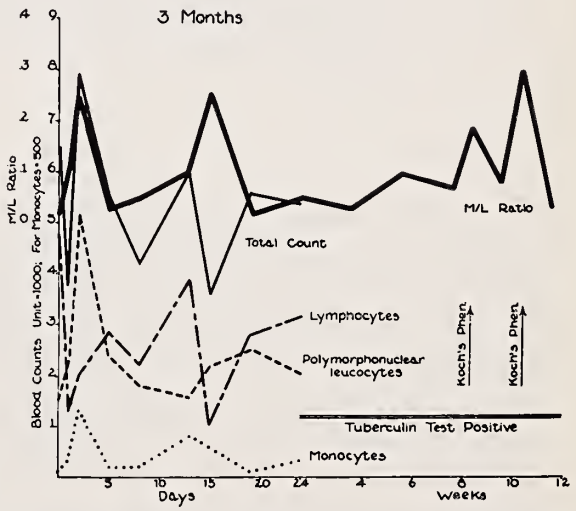
RESULTS

Intravenous Route

Swelling of the septal cells throughout the lung began almost at once and was well pronounced by the 3d hour. The swollen cells protruded into the alveoli as small nodules (fig. 1). A similar histiocytic swelling occurred about the small blood vessels. This nonspecific process remained active during the formation of the tubercle (3–15 days) and then gradually subsided (1 month). However, at 1 month, 3 months, and 1 year, if large doses of tuberculin were injected intradermally (id) (1000 TU—OT), and if a Koch's phenomenon was manifest, there was a marked swelling of the alveolar walls by hyperemia, septal cell swelling, intracapillary lymphocytes, and polymorphonuclear leukocytes (fig. 2). This pronounced anamnestic-type reaction did not occur if the tuberculin test was small (5 × 5 mm). About the time allergy set in (9–15 days), there was a marked infiltration of lymphocytes about the tubercles (fig. 3).

The reaction of the Kupffer cells and the perivascular histiocytes in the liver occurred early, as in the lung. The cells became swollen, the nuclei vesicular, and the cytoplasm basophilic; phagocytosis increased. The number of Kupffer cells increased, forming small nodules (fig. 4). Similarly, the reticulum cells of the spleen, the perivascular histiocytes in the hilus of the kidney, and the interstitial cells in the kidney because swollen and increased. Generally, the pattern and duration of reactions before and after stimulation by the specific antigen (OT) followed those of the lung.

The responses of the blood mirrored those of the tissues. The monocytes reached their highest number after 3 days, thus lagging behind the response noted in the tissue. The lymphocytes first fell and then rose, reaching their height by the 11th or 12th day. The M/L (monocyte/lymphocyte) ratio was higher after 3 days and again after 15 days. After 8–10 weeks, a large dose of tuberculin given id produced a peak in the M/L ratio, evidence for a stimulation of the RES, particularly in the monocytes (text-fig. 1).



TEXT-FIGURE 1.—Blood profile following iv administration of BCG.

Aerosol Route

Histologically, the pattern was similar to that of the iv group, although these studies were less extensive. One month following exposure and forty-eight hours after tuberculin testing (with large reactions—25 × 25 mm to 100 TU), there were a marked stimulation of septal cells of the alveoli and a monocytic-lymphocytic infiltration in the walls, which made the walls 3–5 times the normal thickness (fig. 5). In the liver there were marked proliferation and an increase of the Kupffer cells (fig. 6).

Intradermal Route

There was a slight to moderate swelling of the septal cells of the lung noted best at 24 hours (fig. 7). For the next 6 days, the condition was stable. At 8–14 days (after id tuberculin and a positive reaction), there were capillary hyperemia, septal cell swelling, and an increase in intracapillary cellularity (fig. 8). With a positive tuberculin reaction at 1 year, there was a moderate hyperemia with thickening of the septal walls, resulting mainly from intracapillary cellularity by lymphocytes and monocytes.

The Kupffer cells of the liver were not prominent until the 3d day (fig. 9). At 17 hours, phagocytosis of red blood cells and granules were increased. The increased cellularity remained for a month or so and then receded, but after a positive tuberculin test the cellular reaction was augmented (up to 1 year). The pattern of reaction was similar in the spleen, interstitial cells in the kidneys, etc.

The activity of the blood monocytes, polymorphonuclear leukocytes, and lymphocytes paralleled the activity in the tissues.

Oral Route

There was a transitory thickening of the septal cells of the lung (fig. 10) which disappeared by the 13th or 14th day. No animals developed positive tuberculin tests, so after testing (up to 9½ months) no hyperemia or proliferation of the septal cells was noted. There were a slight rounding of the Kupffer cells at 5 hours and some increase in their number at 4 days along with increased phagocytosis and basophilia of the cytoplasm. After 13 days, these reactions subsided.

Reticulum cell phagocytosis increased somewhat in the spleen, and the interstitial cells of the kidneys were swollen. The sections of the jejunum and ileum were the same as those of normal animals.

Following the ingestion of BCG, all cellular elements of the blood moderately increased. From the 4th to the 6th week, the M/L ratio was higher than that of the controls.

DISCUSSION

In the guinea pig, relatively large doses of BCG via the iv and the AV routes resulted in the most profound stimulation of the lympho-RES in the lungs, liver, spleen, kidneys, and lymph nodes. The oral route gave the least stimulation of the lymph-RES and the id route was intermediate between the iv and AV and the oral routes. The tuberculin reactions were strongly positive in the iv, AV, and id routes, but negative following oral vaccination of suckling guinea pigs.

The generalized response of the lympho-RES was manifest for several weeks only. However, after a large local skin reaction following tuberculin injection, there was again a marked activity of the lympho-RES, noted by swelling of the septal cells of the lungs, Kupffer cells of the liver, reticulum cells of the spleen, and lymph nodes, etc. This anamnestic reaction was seen after 1 month, 3 months, 12 months, and 14 months (duration of expt.) following BCG vaccination. It is only when the host is in this hyperactive state that a nonspecific effect is manifest. It is therefore important to determine the most effective routes of administration in a given species of animal, and the proper dosage of BCG, to obtain the desired nonspecific effect against neoplastic cells. Furthermore, it is necessary to repeat the specific stimulus at regular intervals to maintain a relatively large antigen depot or the nonspecific effect will not be operative.

The specific effect is also manifest after specific stimulation. Thus in medical students vaccinated with BCG by the AV route, inhibitory activity

by a given number of disrupted white blood cells against H37RV in vitro was enhanced after tuberculin testing. Before tuberculin testing, an average of 29,000 cells caused no inhibition of growth, whereas in 4 of 6 samples after tuberculin testing, an average of 14,000 cells caused nearly complete inhibition (*8*) ($P = 0.012$) (table 1).

TABLE 1.—Inhibition of H37RV by disrupted white blood cells 3 months after vaccination with BCG aerosol

Before tuberculin test		After tuberculin test	
Number of cells in 0.2 ml test mixture	Percent growth (control = 100%)	Number of cells in 0.2 ml test mixture	Percent growth (control = 100%)
30,000	88.63	22,500	104.59
27,000	102.66	11,428	0
28,000	67.59	14,000	93.11
32,000	99.93	25,000	0
35,000	90.99	11,111	0
25,000	99.51	10,322	2.23
31,000	105.32		

Many studies have indicated that BCG given prophylactically before challenge will inhibit or completely retard leukemic or tumor grafts (*7, 9, 10*).

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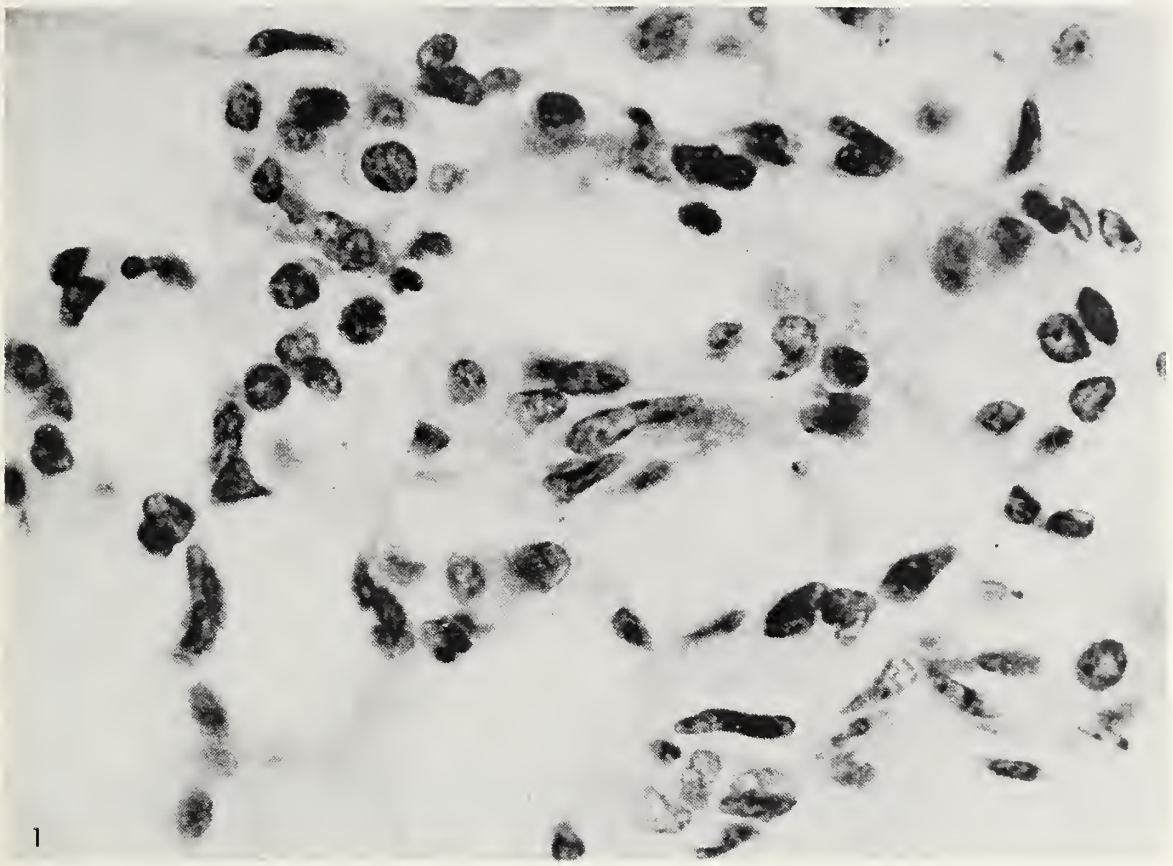

FIGURE 1.—Guinea pig lung (1¼ hr after iv BCG): Septal cells are swollen and arranged along inner aspect of alveolar walls. *Note* flat dark-staining nuclei of the capillary endothelium in contradistinction. Hematoxylin and eosin. × 500

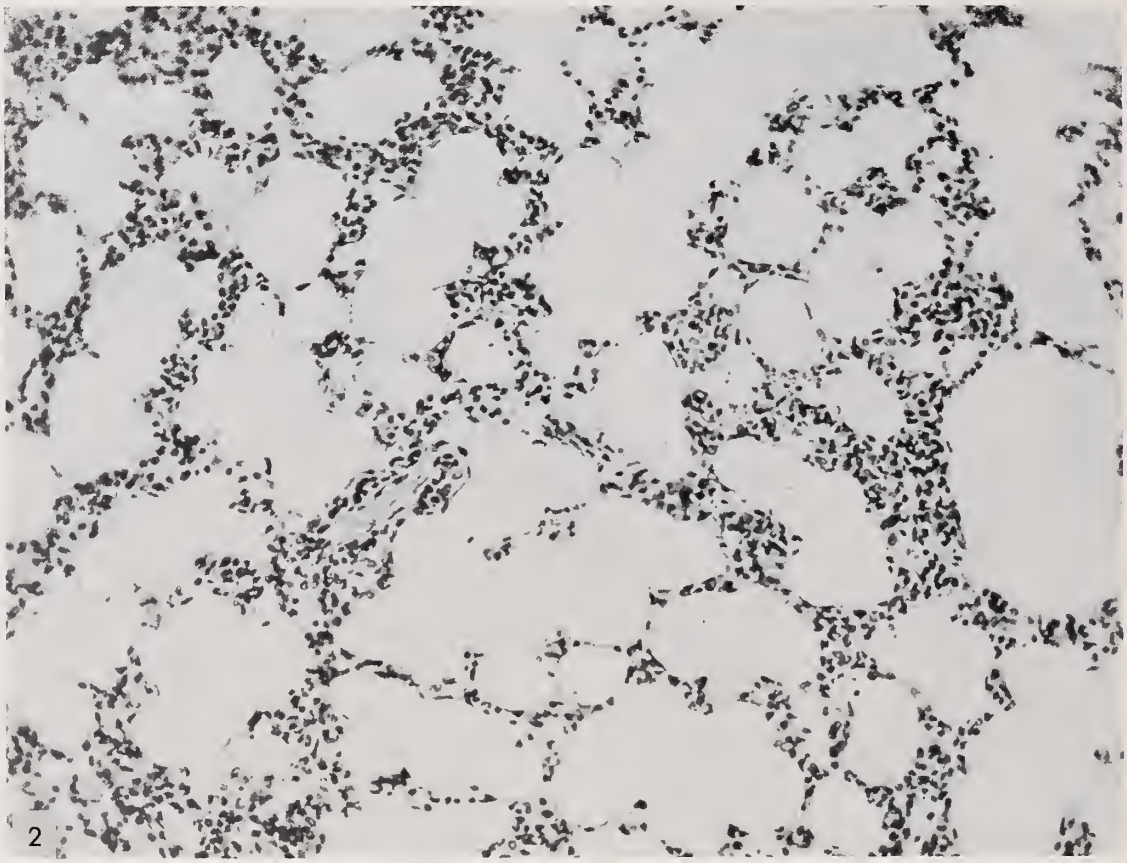

FIGURE 2.—Guinea pig lung (3 months after iv BCG): Moderate hyperemia and septal cell swelling following a Koch's phenomenon due to old tuberculin. Hematoxylin and eosin. × 125

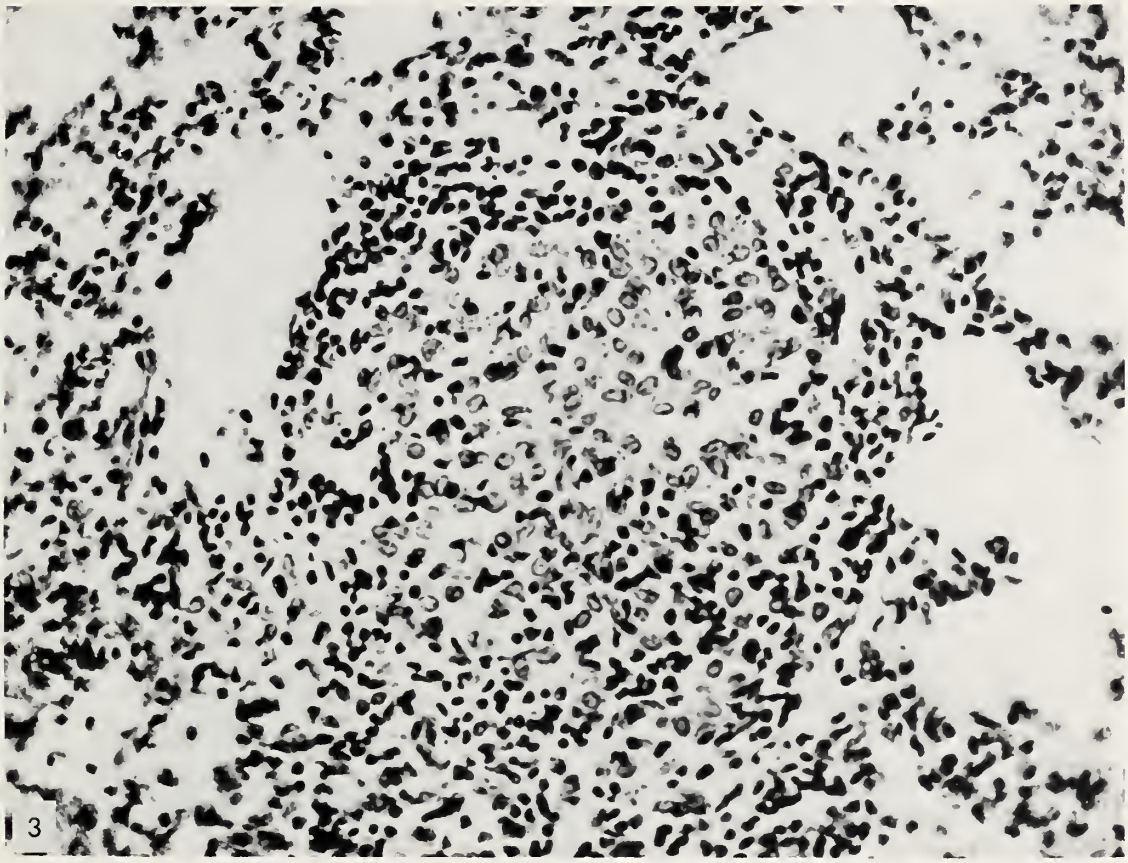

FIGURE 3.—Guinea pig lung (15 days after iv BCG): Tubercle composed of epithelioid cells, surrounded and invaded by lymphocytes. Hematoxylin and eosin. × 250

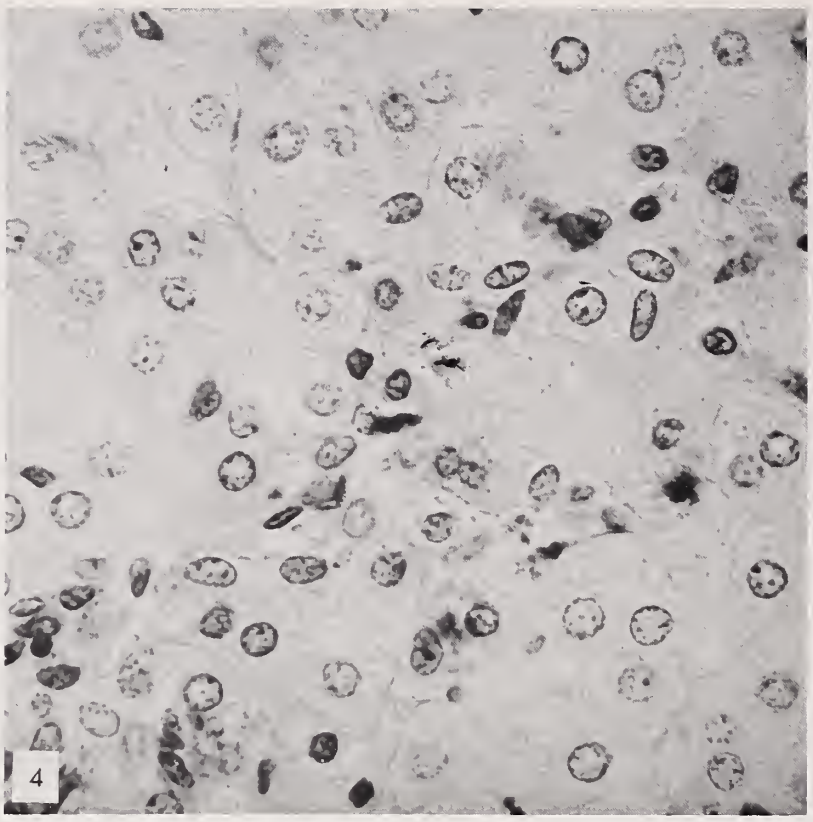

FIGURE 4.—Guinea pig liver (16 hr after iv BCG): Proliferation of the Kupffer cells and their grouping into small nodules. Some of the cells contained tubercle bacilli. Hematoxylin and eosin. × 500

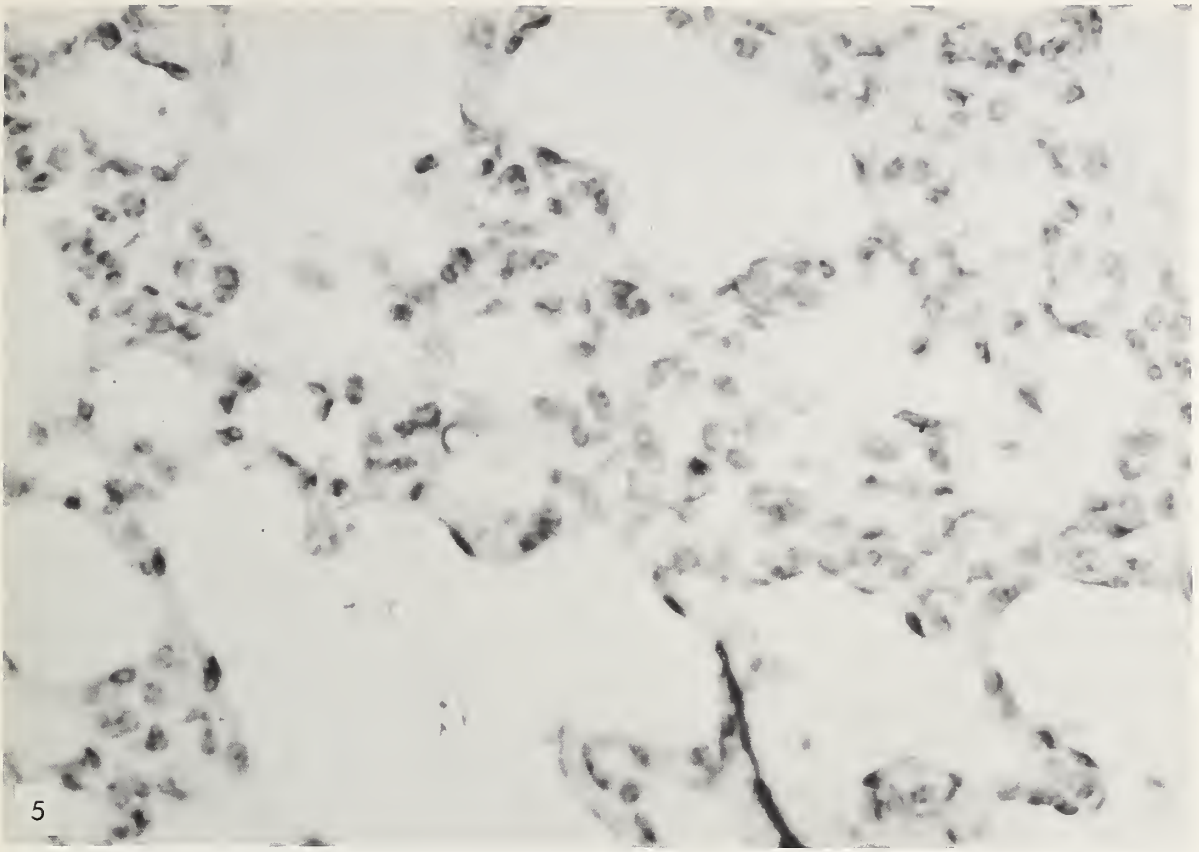

FIGURE 5.—Guinea pig lung (1 month after aerosal BCG) : Capillary walls are swollen as a result of swelling of septal cells and intraseptal cellularity 48 hours after tuberculin testing. Hematoxylin and eosin. × 250.

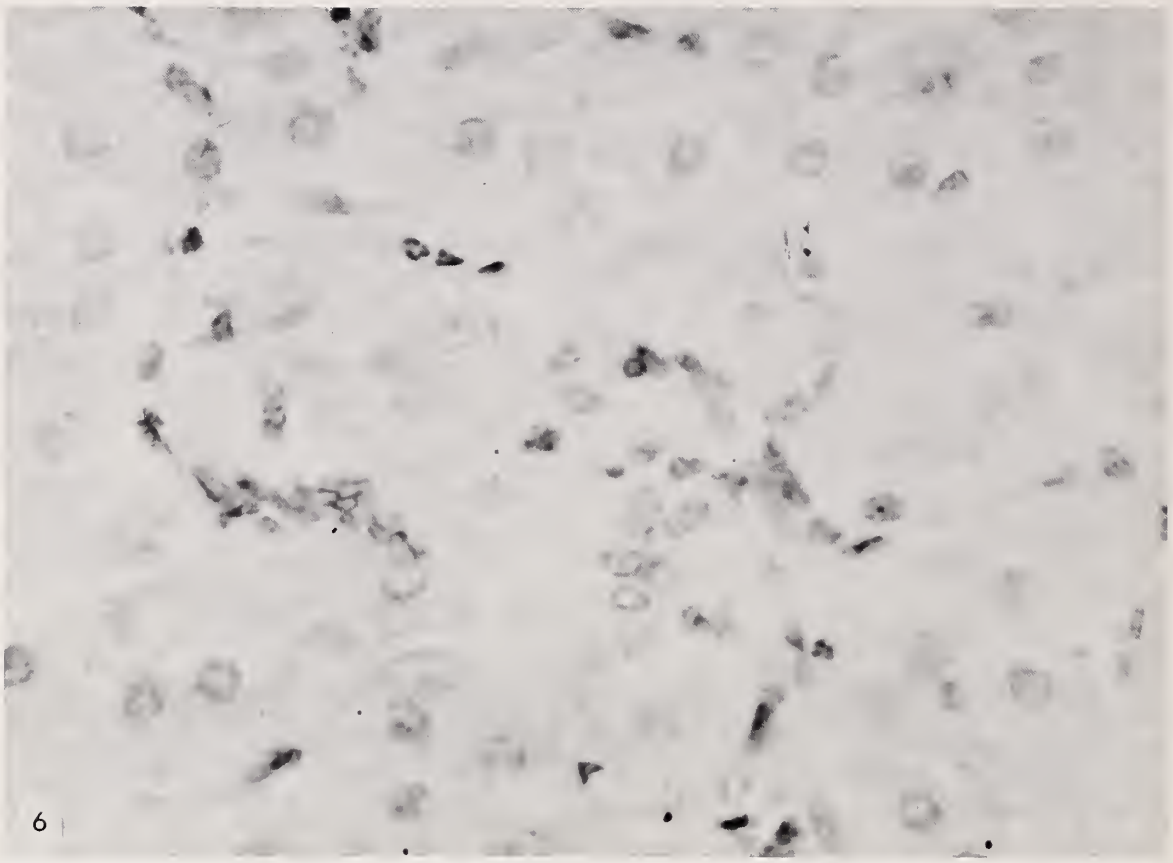

FIGURE 6.—Guinea pig liver (1 month after aerosal BCG): Kupffer cells increased and show increased phagocytosis. Hematoxylin and eosin. × 500

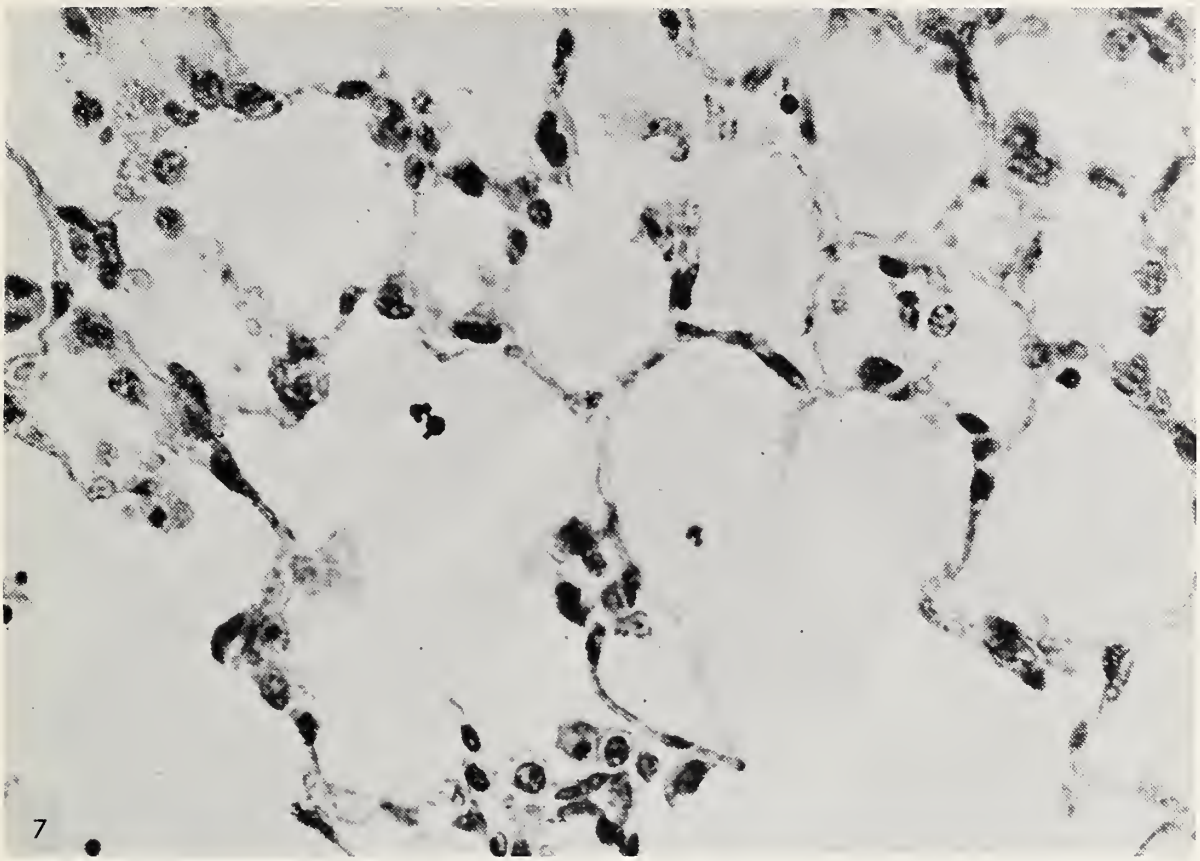

FIGURE 7.—Guinea pig lung (24 hours after intradermal inoculation): Aveolar walls are thickened as a result of septal cell swelling. Hematoxylin and eosin. × 360

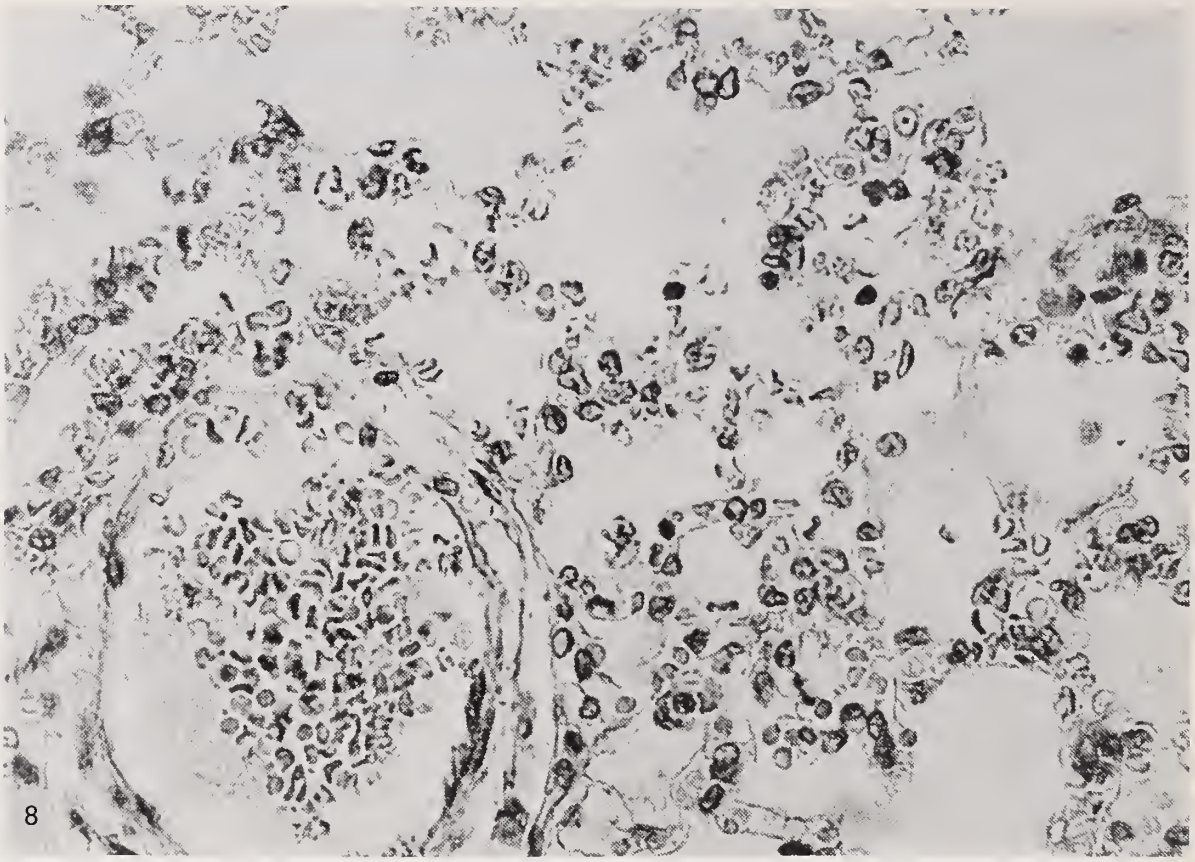

FIGURE 8. Guinea pig lung (14 days after intradermal BCG): Capillaries are diffusely dilated and filled with blood. Tuberculin test was positive on day of sacrifice. Hematoxylin and eosin. × 360

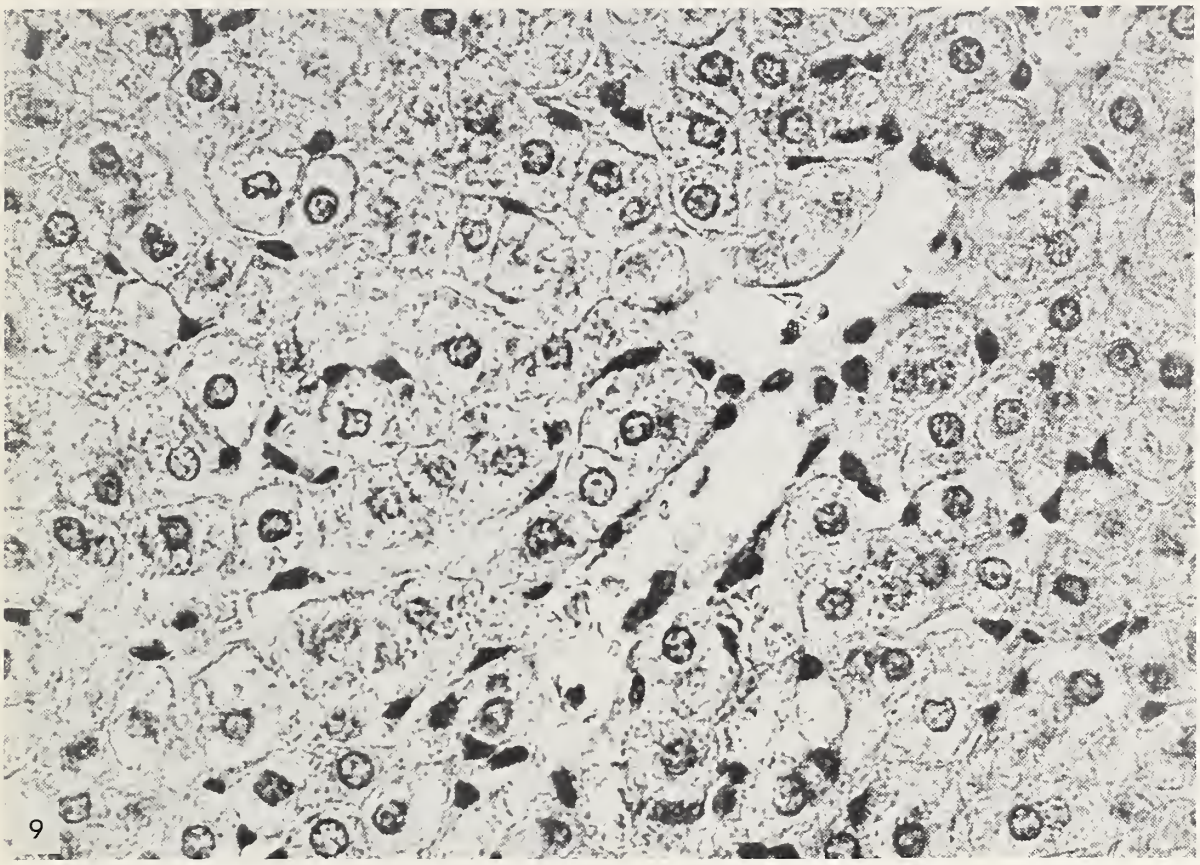

FIGURE 9.—Guinea pig liver (3 days after intradermal BCG): Prominence of Kupffer cells is striking. Clusters of histiocytic cells are common. Hematoxylin and eosin. × 360

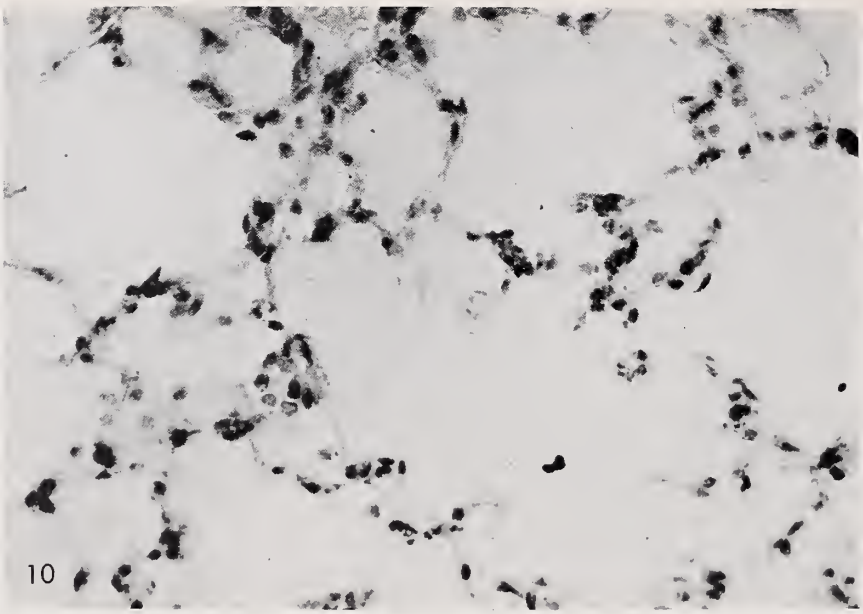

FIGURE 10.—Guinea pig lung (48 hr after last oral dose of BCG): There is still increased cellularity but it is extracapillary. Hematoxylin and eosin. × 360

DISCUSSION

D. W. Weiss: The presentations of Dr. Chaparas, Dr. Mardiney, and Dr. Rosenthal are open to general discussion. I would suggest the first questions focus specifically on details of experimental procedure rather than on philosophy.

T. Borsos: Are all of these animals immunosuppressed?

S. D. Chaparas: Except for the controls. All of them were given 1,3-bis (2-chloroethyl) -1-nitrosourea (BCNU) on day 7; on days 10, 13, and 16, they were challenged with BCG. In other words, this approach is that used in the human situation. When a man has a tumor, what do you do? You treat him with various drugs, and he is immunosuppressed.

B. Zbar: Dr. Chaparas, in what sites in the body does the LSTRA tumor grow In what organs does it multiply?

Chaparas: A systemic leukemia, it lands wherever all leukemia cells land: in the lymph nodes. It is distributed generally. When we suppressed the animals on day 7, the leukemia cells were in the blood. We demonstrated this by passively transferring the tumor. But it was found everywhere.

Borsos: In your dose-response studies in which 800 organisms were found to be as effective as 1 million, what happens if you inject 100 or 50 organisms?

Chaparas: A total of 800 organisms gave 100% survival by this route, and 8 million gave much less survival.

Borsos: Then there still could be a more effective dose.

Chaparas: There still may be a more effective dose, but this is a reverse dose. This is very important, you see. If you use BCG with many organisms by many routes in a highly immunized individual, the BCG treatment may backfire. Antibody production may be blocked.

Weiss: That would seem to be conjecture at this point.

Chaparas: Yes, of course.

P. Alexander: Could you describe the ia?

Chaparas: The organisms were subcutaneously distributed equally into the inguinal and axillary areas of the mice.

Unidentified speaker: Dr. Chaparas, how long did you observe the animals for survival?

Chaparas: The animals were observed for about 80 days; they survived that period of time.

Unidentified speaker: Also in your control groups I assume you had a control that just received BCG?

Chaparas: Yes.

A. Goldin: Dr. Chaparas, maybe I'm not clear on your experimental design, but I think you are giving the BCNU, or the cyclophosphamide, at some time after the LSTRA tumor is put into the animal and you are using the agents as immune suppressants. But both of these are also antitumor agents.

Chaparas: That is right.

Goldin: I don't know how you distinguish between the antitumor activity of these agents and the immune suppressant activity of these agents when the tumor is already in the animal and growing.

Chaparas: The tumor is growing: Nodules are present, and there is systemic spread of the tumor. When you give BCNU, you do get suppression of the growth of tumor cells. As a matter of fact, the cell dips way down, to a point where you can no longer demonstrate it in the blood. And if you inject the BCG early intradermally, you get very good protection. But if you wait until day 16 and you give the same dose intradermally, you get less effective protection. But I'm conjecturing that you do have immunosuppression. We are studying the in vitro responsiveness of the cells of the animals at this time.

J. E. Sokal: Would somebody clarify something that bothers me about this dose-response question? After we inject living organisms into an animal, presumably these organisms are going to proliferate in the animal. Now, what precisely do we mean when we say that we give a dose of 1,000, 10,000, or 100,000 organisms? And does this kind of quantitation make sense?

Chaparas: This is precisely the point. Now, we are dealing with many different strains of BCG. We have the French strains, the Canadian strains, etc., and they have different properties. The virulence of these BCG organisms in the different species varies. N. Sher, in my laboratory, is conducting studies right now, and K. Bunch-Christensen, from Copenhagen, has studied the virulence of these organisms. These organisms multiply at different rates. You can show a dose response. As you grade the dose, the spleen gets larger. The organisms multiply to different degrees at a certain time. If you take the spleen or the liver, grind it up, and count, you can quantitate this effect. Now, is the virulence of the organism important in immunotherapy? Virulence of BCG might be counteractive to immunotherapy.

Weiss: We are conjecturing too much again, because the specific question was . . .

Chaparas: The specific question is—and we're trying to answer it and we have data on it—that the organisms multiply at different rates depending on the strain used, and you can quantitate these rates by doing actual counts.

Sokal: Suppose you inject 1,000 organisms. How many will you have 24 hours later?

Chaparas: Within the 1st week, you probably won't find any increase in organisms; but at days 10–13, you will find a slight increase. At day 28, you might find an increase of several logs in the spleen.

G. B. Mackaness: In the mouse you get a maximum increase of BCG of about 2 logs, which takes about 12–14 days to reach.

Goldin: In an immunosuppressed animal, do you get only a 2-log increase?

Mackaness: The rate at which the organism grows is independent of the state of immunity of the animal during the first 10 days. In the immunosuppressed animal—at least in the thymectomized animal—the proliferation of the organism goes on not indefinitely, but it does go on for about 28 days.

Weiss: I might point out, Dr. Goldin, with regard to this, that there is some good and solid work by C. H. Pierce, W. B. Schaefer, and R. J. Dubos published in the middle 1950's in the *Journal of Experimental Medicine,* following the kinetics of multiplication of several strains of BCG in a variety of mouse organs. Are there other comments?

Chaparas: I have one comment, Dr. Weiss, and it's in the form of a plea, actually. When people are using different manufacturers' BCG's, which vary in the number of bacteria and the route of administration, and people don't report the numbers of organisms in each, this is detrimental to progress. People should try to give as much detail and as many criteria as possible before they start drawing conclusions: This will become evident in tomorrow's discussion.

BCG in Cancer Immunotherapy: Results Obtained with Various BCG Preparations in a Screening Study for Systemic Adjuvants Applicable to Cancer Immunoprophylaxis or Immunotherapy[1,2]

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SUMMARY—Ten preparations of BCG were submitted to the 5 tests of our *immunity systemic adjuvant screening battery*. BCG was injected intravenously into experimental animals at day 14 before the immune machinery and the antigen met. Only the *"French fresh" Pasteur Institute preparation* gave a significant result, which indicates an *immune stimulation with all tests:* hemolytic plaque-forming cell test (Jerne), graft-versus-host reaction, prophylaxis of L1210 leukemia, solid Lewis tumor, and ICIG Ci_1 solid tumor. The other lyophilized preparations, including the French one, gave dissociated results or even results that were contradictory from one test to another. The reasons are unknown. No intravenously injected BCG preparation enhanced the growth of any tumor.—Natl Cancer Inst Monogr 39: 107–112, 1973.

AS KNOWN for 15 years, and confirmed on various experimental models, when BCG is administered intravenously (iv) or intraperitoneally (ip) *before* inoculation with a bacteria (*1*) or with allogeneic or isogeneic tumor cells (*2–5*), it produces *immunoprophylaxis* of the infection or of the neoplasm. The reason usually accepted for this effect was stimulation of the phagocytic capacity of the host (*6*).

We recently showed that BCG, injected iv *after* inoculation with an isogeneic leukemia (L1210) known to carry tumor-associated antigens (*7*), cured a noticeable percentage of the animals, provided: *a*) The number of tumor cells was $\leq 10^5$ (*8*) and *b*) BCG was combined with irradiated tumor cells (ITC) as specific immune stimulation (*5, 8*). The ITC alone did not cure any animals, although they retarded mortality; and BCG alone, in the first experimental model, did not delay mortality significantly. We thus concluded that *active immunotherapy,* which is the immune stimulation applied to the treatment of an established tumor, requires that the following conditions be met: *a*) *BCG must be combined with ITC,* and *b*) the tumor volume must have been previously reduced by other treatment (s). We dedicated our first clinical trials to eradicating *the minimal residual disease* left by previous chemo-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Presented in the absence of Professor G. Mathé by R. Weiner, M.D.

[3] INSERM and Association Claude-Bernard.

[4] 14–16, avenue Paul-Vaillant-Couturier.

therapy in acute lymphoid leukemia (ALL). The first trial (*9*) demonstrated the effectiveness of active immunotherapy in human ALL, and its importance has been underlined by a review of 100 patients (*10*). The effectiveness of active immunotherapy also was recently confirmed in acute myeloid leukemia by Hamilton-Fairley et al. (*11*).

This kind of active immunotherapy is *systemic,* hence different from the more recently introduced *local* immunotherapy that consists of intratumoral injection with BCG (*12, 13*).

Because it is difficult to apply BCG to human patients, we set up a screening battery to detect other *systemic immunity adjuvants* (*14*) *that* could be administered more easily with a more scientific and quantifiable method. Results after 5 tests of this battery of 10 agents (*15*)—among which were a preparation of BCG ("French fresh"), various nonsoluble or soluble extracts of BCG, several preparations of *Corynebacterium parvum* or of *C. granulosum,* poly I:C, and poly A:U— showed that fresh preparations of the BCG French strain provided by the Pasteur Institute were the most strongly and frequently active.

Moreover, this study in which different routes of administration were compared demonstrated that: *a*) the iv route was the only efficient one for the BCG with the Jerne test, a reaction involving T- and B-lymphocytes (*16*), and with the graft-versus-host (GVH) reaction, which involves only T-lymphocytes (*17*); *b*) the iv route delayed mortality when used for the immunoprophylaxis of 3 grafted murine tumors, but the subcutaneous (sc) route enhanced the growth of one of them, the Lewis tumor.

In our clinical trials (*9, 10*), we used fresh preparations of the French strain BCG provided by the Pasteur Institute. Other groups using other strains published negative results (*18*). The failures in their protocols can be explained by the use of BCG alone instead of in combination with ITC (which is not in accord with our basic experimental model); another explanation for their failures might be the potentially different values of the various BCG preparations. One can point to the remarkable results of Bluming et al. (*19*), who stimulated immune reactions and prolonged the remissions of melanoma patients with the Pasteur lyophilized BCG, but failed in these two objectives with the Glaxo strain.

For these reasons we submitted 10 different preparations of BCG to our screening battery. The tests were simplified in comparison with those used in the previous study of the 10 agents (*15*) in that only iv injections were given. This simplification was motivated by financial considerations and by the results of the first study which showed this route was consistently the most active.

MATERIALS AND METHODS

Materials.—The 10 preparations studied are listed in table 1. The "French fresh" preparation was provided by the Pasteur Institute, the "Glaxo" strain by Glaxo Laboratories L, and the "Chicago" by the Research Foundation. All the other preparations were obtained from the Statens Serum Institut.[5] All preparations were lyophilized except the "French fresh."

The number of "viable units" per mg differs from one preparation to another, varying from 1.8×10^6 (Prague 725) to 66.5×10^6 (Japanese 172). These numbers are not very meaningful in practice, because a "viable unit" corresponds more often to a clump than to a single bacteria. For this reason we injected 1 mg of material based on the manufacturer's report of initial packaged weight regardless of whether it was subsequently kept fresh or lyophilized. Table 1 shows other characteristics, such as virulence (in golden hamster), germination rate, and opacity as published by Bunch-Christensen et al. (*20, 21*).

Methods.—The tests of our screening battery and the reasons for their choice were described in (*15*). Table 2 lists the specific methods in which they were employed. Only iv injections were given at day 14 before the "immune machinery and the antigens met," regardless of what the latter might be—sheep red blood cells (SRBC) as in the Jerne test or tumor-associated antigens as in the tumor tests. The BCG's were injected into the donors 14 days before the lymphocytes were transformed in the GVH reaction.

For the Jere test, the plaques were counted at day +4 after the ip injection of SRBC. For the statistical control of the results, the Student-Fisher test was used.

For the GVH test, the mortality was studied and the index established:

$$\text{Index} = \frac{\text{median survival of experimental animals}}{\text{median survival of the controls}}$$

For the statistical control, the Wilcoxon test was used.

[5] We thank Miss K. Bunch-Christensen for providing us with the test material and for technical data on its packaging.

TABLE 1.—Various preparations and/or strains of BCG submitted to "ICIG systemic immunity adjuvant screening" for comparison

Preparations and/or strains	Obtained from	"Viable units" per mg	Other characteristics*		
			Virulence for golden hamster	Germination rate (% at 24 hr)	Opacity (suspension of 0.75 mg/ml)
French (fresh)	Pasteur Institut	7×10^6			
French 1173 P_5 (lyophilized)	Statens Serum Institut	6.2×10^6	++	75	0.35
Glaxo "	Glaxo Laboratories L	60×10^6	+	50–75	0.35
Chicago "	Research Foundation	4.2×10^6			
Brazilian (Moreau) "	Statens Serum Institut	9×10^6	++++	75–90	0.40
Madras 809 "	Statens Serum Institut	4×10^6	+	75–90	0.38
Swedish "	Statens Serum Institut	16×10^6	++	75	0.35
Prague 725 "	Statens Serum Institut	1.8×10^6	+	75	0.33
Danish 1331 "	Statens Serum Institut	6.3×10^6	+++	75	0.36
Japanese 172 "	Statens Serum Institut	66.5×10^6	++	75	0.38

* Data from the literature (*20*, *21*).

For the L1210 leukemia, mortality was recorded; for the statistical control, the Student-Fisher test was used.

For the solid tumors, mortality was studied; for the statistical control, the Wilcoxon test was used.

All animals were evaluated for the results of the experiments in double-blind fashion.

TABLE 2.—Tests and methods used

Tests	Day 0	Injection of adjuvants	Results
Hemolytic plaque-forming cell (HPFC) test (Jerne)	10^9 SRBC, ip	Day −14	Plaque-forming cells at day +4 after SRBC injection
GVH	10^7 Bone marrow and 2.5×10^7 lymph node C57BL/6 cells to $(C57BL/6 \times DBA/2)F_1$	Day −14 (to the donors)	Mortality
L1210 leukemia	10^3 Leukemic cells, iv	Day −14	Mortality
Solid Lewis tumor	2×10^6 Tumor cells, sc	Day −14	Mortality
Solid ICIG Ci_1 tumor	2×10^6 Tumor cells, sc	Day −14	Mortality

RESULTS

The results are given in tables 3 through 7.

Table 3 shows that only "French fresh" BCG significantly increased the number of HPFC in the Jerne test. The Glaxo BCG decreased it, despite a considerable increase in the spleen weight. In view of the first result with this strain, the test was repeated and results were identical. The other preparations did not modify the indices.

The effect of the different BCG's on the GVH is indicated in table 4. Only "French fresh" BCG significantly accelerated mortality. All the lyophilized BCG's delayed it, except that the Brazilian, the Madras, and the Danish exerted no significant modification.

TABLE 3.—Comparison of effect of various BCG strains on HPFC test (Jerne) (1 iv injection of BCG 14 days before ip injection of SRBC)

Strains	Effect	Index*	Statistics†
French (fresh)	↗	2.8	S at 2% ($t = 2.9$)
French 1173 P_5 (lyophilized)	↘	1.1	NS
Glaxo "	↘	0.3	S >1‰ ($t = 4$)
Chicago "		1.1	NS
Brazilian (Moreau) "		1.1	NS
Madras 809 "		1.1	NS
Swedish "		1.1	NS
Prague 725 "		1.08	NS
Danish 1331 "		1.1	NS
Japanese 172 "		1.09	NS

* Index = $\frac{\text{mean No. of plaque-forming cells/spleen of tested mice}}{\text{mean No. of plaque-forming cells/spleen of controls}}$.

† Statistics = Student-Fisher test. S = significant; NS = not significant.

TABLE 4.—Comparison of effect of various BCG strains on GVH lethality (1 iv injection of BCG to donor C57BL/6, 14 days before lymphocyte iv injection to (DBA/2×C57BL/6)F_1)

Strains		Effect	Index*	Statistics†
French (fresh)		↗	0.21	S at 2% ($\epsilon = 2.41$)
French 1173 P_5 (lyophilized)		↘	1.35	S >1% ($\epsilon = 2.9$)
Glaxo	"	↘	1.35	S at 1% ($\epsilon = 2.5$)
Chicago	"	↘	1.35	S >1% ($\epsilon = 2.9$)
Brazilian (Moreau)	"		1.30	NS
Madras 809	"		1	NS
Swedish	"	↘	2	S at 2% ($\epsilon = 2.36$)
Prague 725	"	↘	2	S >1% ($\epsilon = 3.1$)
Danish 1331	"		1.14	NS
Japanese 172	"	↘	1.57	S >1% ($\epsilon = 2.8$)

* Index = $\frac{\text{median survival by experimental animals}}{\text{median survival of the controls}}$.

† Statistics: Wilcoxon's nonparametric test. S = significant; NS = not significant.

Table 5 indicates the results obtained in the prophylaxis of L1210 leukemia. Not only the "French fresh" but also the lyophilized French, Glaxo, Brazilian, Danish, and Japanese preparations significantly prolonged survival. The other lyophilized preparations did not modify it.

Table 6 indicates the results obtained on the prophylaxis of the solid Lewis tumor. Only the "French fresh" and the Chicago preparations significantly prolonged survival. All the others, except the Brazilian and Madras, tended to shorten survival—but not significantly.

Table 7 shows the effect of the 10 preparations on the prophylaxis of the ICIG Ci_1 solid tumor. The "French fresh" and the lyophilized Glaxo, Chicago, and Danish BCG's delayed mortality significantly. The lyophilized French preparation did not modify it. All the others tended to accelerate mortality—but not significantly.

TABLE 5.—Comparison of effect of various BCG strains for immunoprophylaxis of L1210 leukemia (1 injection of BCG 14 days before iv inoculation of 10^3 leukemia cells)

Strains		Effect	Index*	Statistics†
French (fresh)		↗	1.6	S at 2% ($t = 3.55$)
French 1173 P_5 (lyophilized)		↗	1.15	S at 1% ($t = 3.3$)
Glaxo	"	↗	1.27	S at 1‰ ($t = 10.6$)
Chicago	"		1	NS
Brazilian (Moreau)	"	↗	1.27	S at 1‰ ($t = 9$)
Madras 809	"		1	NS
Swedish	"		1	NS
Prague 725	"		1	NS
Danish 1331	"	↗	1.15	S at 5% ($t = 2.68$)
Japanese 172	"	↗	1.15	S at 1% ($t = 3.58$)

* Index = $\frac{\text{median survival of experimental animals}}{\text{median survival of the controls}}$.

† Statistics = Student Fisher test. S = significant; NS = not significant.

TABLE 6.—Comparison of effect of various BCG strains for immunoprophylaxis of Lewis solid (tumor 1 iv injection of BCG 14 days before sc inoculation of 2×10^6 cells)

Strains		Effect	Index*	Statistics†
French (fresh)		↗	2.6	S at 1% ($\epsilon = 2.90$)
French 1173 P_5 (lyophilized)			0.9	NS
Glaxo	"		0.9	NS
Chicago	"	↗	2	S >1‰ ($\epsilon = 3.30$)
Brazilian (Moreau)	"		1	NS
Madras 809	"		1	NS
Swedish	"		0.9	NS
Prague 725	"		0.9	NS
Danish 1331	"		0.9	NS
Japanese 172	"		0.9	NS

* Index = $\frac{\text{median survival of experimental animals}}{\text{median survival of the controls}}$.

† Statistics: Wilcoxon's nonparametric test. S = significant; NS = not significant.

TABLE 7.—Comparison of effect of various BCG strains for immunoprophylaxis of solid tumor ICIG Ci_1— (1 iv injection of BCG 14 days before sc inoculation of 2×10^6 cells)

Strains		Effect	Index*	Statistics†
French (fresh)		↗	1.19	S at 1% ($\epsilon = 2.5$)
French 1173 P_5 (lyophilized)			1	NS
Glaxo	"	↗	2	S >1‰ ($\epsilon = 3.7$)
Chicago	"	↗	2	S >1‰ ($\epsilon = 3.7$)
Brazilian (Moreau)	"		1.15	NS
Madras 809	"		1.12	NS
Swedish	"		1.15	NS
Prague 725	"		1.15	NS
Danish 1331	"	↗	1.25	S at 2% ($\epsilon = 2.34$)
Japanese 172	"		1.12	NS

* Index = $\frac{\text{median survival of experimental animals}}{\text{median survival of the controls}}$.

† Statistics: Wilcoxon's nonparametric test. S = significant; NS = not significant.

DISCUSSION

In summary, only the "French fresh" BCG gave a significant result consistent with an immunostimulating action on all tests (table 8). This effect was favorable against the 3 tumors. We mentioned in (*15*) that sc administration of this fresh BCG can enhance the growth of the Lewis tumor.

The lyophilized preparations either: *a*) induce different immune modifications, according to the various tests, such as an inhibition of GVH (Glaxo, Chicago, Japanese, plus a decrease of HPFC for the Glaxo), but retard mortality for one or several tumors; *b*) do nothing, as on the Jerne and GVH tests, and inhibit growth of a tumor (Brazilian, Danish): *c*) reduce the GVH but do not affect tumor growth (Swedish, Prague); or *d*) as the Madras, do not exert any significant action on any test.

We have no data at present to explain the differences in action of some BCG's in the various tests. Probably what is measured in each one is the sum of several possible effects. In fact, a systemic immunity adjuvant can stimulate or inhibit an immune reaction, depending on various parameters (*15*).

One wonders also if one of these effects cannot be followed by another. This suggests, in this case, that if all BCG preparations were injected iv at the same time and in the same dose, the differences might be due to differences in kinetics, either of the liberation of the active immunostimulating molecule from the bacterias by the reticuloendothelial system or of the destruction of it.

In a cooperative work now in progress at our Institute, the respective effects of an iv BCG injection on T- and B-lymphocyte-specific immune reactions and on their circulations and on macrophage functions are being investigated to elucidate parameters that can explain the data recorded here.

From practical and clinical viewpoints, it is satisfactory to note that no BCG preparation administered iv has enhanced any tumor growth, whereas we showed previously (*15*) that sc injected BCG and other adjuvants injected by various routes can shorten survival of solid tumor-carrying animals.

TABLE 8.—Summary of results

Strains of BCG	Jerne test	GVH test	Immunoprophylaxis of: L1210	Lewis tumor	ICIG Ci_1
French (fresh)	I* = 2.8 S† 2%↗	I‡ = 0.21 S§ 2%↗	I‡ = 1.6 S† 2%↗	I‡ = 2.6 S§ 1%↗	I‡ = 1.19 S§ 1‰↗
French 1173 P5 (lyoph.)	I = 1.1 NS	I = 1.35 S 1%↘	I = 1.15 S 1%↗	I = 0.9 NS	I = 1 NS
Glaxo (lyoph.)	I = 0.3 S 1%↘	I = 1.35 S 1%↘	I = 1.27 S 1%↗	I = 0.9 NS	I > 2 S 1‰↗
Chicago (lyoph.)	I = 1.1 NS	I = 1.35 S 1%↘	I = 1 NS	I > 2 S 1‰↗	I > 2 S 1‰↗
Brazilian (Moreau) (lyoph.)	I = 1.1 NS‰	I = 1.30 NS	I = 1.27 S 1‰↗	I = 1 NS	I = 1.15 NS
Madras 809 (lyoph.)	I = 1.1 NS	I = 1 NS	I = 1 NS	I = 1 NS	I = 1.12 NS
Swedish (lyoph.)	I = 1.1 NS	I > 2 S 2%↘	I = 1 NS	I = 0.9 NS	I = 1.15 NS
Prague 725 (lyoph.)	I = 1.08 NS	I > 2 S 1%↘	I = 1 NS	I = 0.9 NS	I = 1.15 NS
Danish 1331 (lyoph.)	I = 1.1 NS	I = 1.14 NS	I = 1 NS	I = 0.9 NS	I = 1.25 S 2%↗
Japanese 172 (lyoph.)	I = 1.09 NS	I = 1.57 S 1%↘	I = 1.15 S 1%↗	I = 0.9 NS	I = 1.12 NS

* $I = \frac{\text{mean No. of PFC/spleen of the tested mice}}{\text{mean No. of PFC/spleen of controls}}$.

† Statistics: Student Fisher test. S = significant; NS = not significant.

‡ $I = \frac{\text{median survival of experimental animals}}{\text{median survival of the controls}}$.

§ Statistics: Wilcoxon's nonparametric test.

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DISCUSSION

J. U. Gutterman: The French BCG has, I believe, a 2-week shelf life. How soon after manufacture do you use it? Within 24 hours?

R. Weiner: We like to use it at the Institute 1 week before it expires, but we use it up to the day it expires.

D. W. Weiss: Did you attempt to equate the time of testing with the time of manufacture of the various preparations for viability and for these various parameters? Or perhaps—I hate to make the suggestion—the French product might have been tested within a day or 2 after production and some of the other products a few months later?

Weiner: That may very well have happened. There was no attempt to control this situation. But the only fresh preparation was the French. The other preparations were received in lyophilized form and stored according to the manufacturer's suggestion.

L. Chedid: I am not going to defend the French strain, but I would like to stress that similar results can be obtained with killed organisms. These preparations do not exhibit the variations sometimes observed with live organisms.

One cause of variability which has not been mentioned today may stem from fermentation problems. Thus, with the same strain, if cultured in flasks or fermentors, one may get material which can be more or less active from one preparation to another.

The advantage of the viable strains resides in the fact that, by division, they greatly and continuously stimulate the host. Nevertheless, these same responses can be evoked by killed organisms if sufficient amounts have been administered.

B. G. Leventhal: It was my impression that the shelf life as labeled in the French lyophilized material was excessively long, and the material actually lost its potency before the end of its prescribed shelf life. Do you have any data on viable organisms—reconstituting the material when it is 1 week old, 2 weeks old, 4 weeks old?

Weiner: Do you mean to say French lyophilized strain?

Leventhal: Yes.

Weiner: I have no data.

Leventhal: Do you have data for any of these organisms?

Weiner: No.

Leventhal: Does Dr. Rosenthal have any information?

S. R. Rosenthal: I recommend that once our vaccine is resuspended it should be used within a couple of hours because after that time viability may be lost. In the lyophilized state, the vaccine will keep for 1 year or longer at 5° C.

M. Chase: This subject seems more important than the immediate issue of whether organisms will suffice in 5 different tests. The question is—and is still unresolved in my mind—must the BCG be living? Dr. Mackaness mentioned to me serious discrepancies between the dead population and the living one in a series of BCG preparations. So if it is antigenic mass one must think of rather than just the number of living viable units, all this information is pertinent to what we are discussing.

Today and tomorrow we will hear more about whether cells can be living. The cell walls reported on by Dr. Ribi are not living. If so, it is the total antigenic mass one must think of. These figures from Dr. Mathé of viable units per milligram have to be brought up against the dead population in the same vial.

Weiner: Dr. Mathé's tests were done with 1 mg of material, irrespective of the viable units. The table was for your reference, so that if you want to take viable units seriously, you could recalculate the data.

Weiss: But, if that is the point, then 1 mg of several different strains with different degrees of viability is a very different milligram within a few days after injection. A static reference to starting quantity is meaningless. You can put in 1 mg and wind up with 10 or with 0.1 within 72 hours!

Weiner: There is a fairly good similarity between the strains as far as viable units go, which showed, nevertheless, a great difference in the physiologic parameters.

K. Bunch-Christensen: Lyophilized products are stable, for if you examine your vaccine immediately after preparation or 1 year after preparation, you will find that viability (in viable units 1 mg or ml) stays the same. There is no change as long as the vaccine is freshly resuspended.

B. B. Mackaness: The main reason for the variation in the viable count per unit mass is due to dead bacterial cells in some preparations, which range between 99.9% and 5% in the preparations of lyophilized vaccines that we've studied. One can determine by doing a total bacterial count with a viable count, and the best lyophilized products that we have examined are only about 5% viable.

Mycobacterial Cell Wall Components in Tumor Suppression and Regression[1]

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SUMMARY—We determined the ability of certain structural elements of the BCG cell wall to suppress tumor development and effect regression of established tumors in the guinea pig. When 37.5 μg untreated cell wall attached to oil droplets was injected intradermally (id) into strain-2 guinea pigs together with 10^6 ascites line-10 tumor cells, tumor growth was suppressed in 3 of 4 animals. A similar dose of cell wall skeleton preparation attached to oil droplets and injected id with 10^6 cells was completely effective, and 18.75 μg cell wall skeleton suppressed tumor growth in 2 of 4 animals. A toxic lipid extracted from the cell (P_3), attached to oil droplets, did not suppress tumor growth, but when administered id with tumor cells, delayed tumor growth 2–3 weeks. A vigorous inflammatory response to id administered P_3 occurred within the first 24 hours. Animals that survived were challenged id after 10 weeks with 10^6 tumor cells contralateral to the side on which they were immunized; all showed systemic immunity to the tumor. Both P_3 and cell wall skeleton were required to cause regression of established line-10 tumors. While tumor immunity may not have been necessary for regression of established tumor, a constant association was shown.—Natl Cancer Inst Monogr 39: 115–119, 1973.

INTRADERMAL GROWTH of a transplantable hepatocarcinoma in syngeneic guinea pigs was suppressed if the tumor cells were injected together with BCG cell walls attached to oil droplets and animals in which tumor growth was suppressed acquired systemic tumor immunity (*1*). Likewise, injection of BCG cell walls attached to oil droplets into established tumors caused the tumors to regress and prevented the growth of metastases (*2*). In both test systems, cell walls were as effective as viable BCG.

When injected into mammals, the BCG cell wall elicits various host reactions, including formation of granulomas, induction of delayed-type hypersensitivity, stimulation of antituberculosis immunity, and, when incorporated with an antigen, powerful adjuvant action. Certain extracts of the tubercle bacillus on repeated intraperitoneal injections into mice, cause death and are thus called "toxic." Both toxic and nontoxic ex-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] National Cancer Institute.

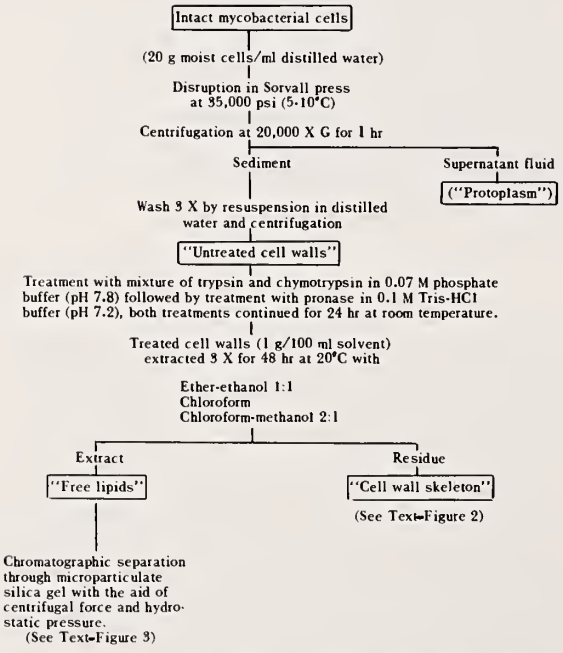
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

tracts, as well as extraction residues, have been described as possessing antitumor activity (*3–6*) and, superficially at least, the findings are confusing. We attempt to clarify them here by studying certain well-defined cell wall structural elements to determine their ability to suppress tumor development and effect regression of established tumors in guinea pigs.

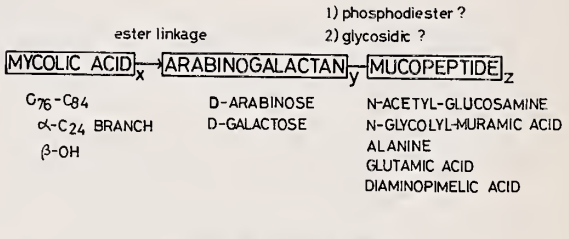
MATERIALS AND METHODS

The procedure for fractionating the mycobacterial cell is outlined in text-figure 1. We prepared cell walls by disrupting cells from the Pasteur Institute strain of BCG in the Sorvall refrigerated pressure cell (*7*). "Free lipids" are most effectively removed from the cell wall by repeated extraction with a series of nonhydrolytic organic solvents according to the method of Azuma et al. (*8, 9*). To facilitate the removal of these lipids, which amounted to about 30% of the untreated cell wall, the cell walls were first deproteinized with trypsin, chymotrypsin, and pronase and then extracted repeatedly with ether and ethanol (1:1), chloroform, and chloroform and methanol (2:1).

Our concept of the cell wall skeleton—the cell wall structure after removal of the free lipids—is shown in text-figure 2. It is an insoluble polymer (i.e., peptidoglycolipid) consisting of a long-chain fatty acid, a polysaccharide, and a mucopeptide. The skeleton also contains a trace of protein whose amino acids differ from those associated with the mucopeptide moiety. Presumably, the methanol-extraction residue from BCG, described by Weiss et al. as having antitumor activity (*3*), consists predominantly of cell wall skeleton. The water-soluble adjuvant and antitumor-active MAAF (mycobacteria adjuvant and antitumor fraction), prepared by Hiu (*5*) and described as a polysaccharide-mucopeptide complex of low molecular weight, is thought to be a product of degradation of the cell wall skeleton.



TEXT-FIGURE 1.—Flow chart demonstrating fractionation procedure used to obtain free lipid extract and cell wall skeleton.

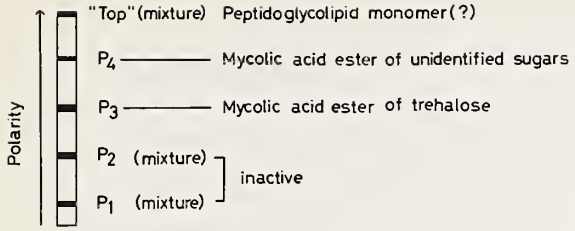


TEXT-FIGURE 2.—Structure of cell wall skeleton existing in the intact cell wall of the tubercle bacillus as an insoluble complex polymer.

In contrast to the untreated cell wall, the cell wall skeleton did not enhance mouse immunity to airborne infection with virulent tubercle bacilli. However, it did suppress the growth of line-10 tumor cells when mixed with these cells and injected into the skin of strain-2 guinea pigs (*see* "Results").

Centrifugal microparticulate gel chromatography revealed that the free-lipid extract contained numerous components in variable quantities, most of which remained unidentified (*10, 11*). The components P_1 and P_2 did not protect mice against infection by virulent tubercle bacilli, whereas P_3 (text-fig. 3) plus a cell wall skeleton product did. P_3 was therefore studied more thoroughly. This component, a mycolic acid esterified to trehalose, appears to be the active principle in classical cord factor preparations, which according to our criteria have been invariably heterogeneous (*12*). P_3 was highly toxic for mice but did not protect them against airborne infection with virulent tubercle bacilli (*12*). However, when recombined with the cell wall skeleton, it did afford protection. This protection was accompanied by an extensive granulomatous reaction in the lungs. In the extract of free lipids, we isolated another mycolic acid ester of slightly higher polarity than P_3 and with similar granuloma-forming activities; it was designated P_1 (Ribi et al., unpublished data).

After removal of P_3 and P_1 from the free-lipid extract, the remaining top material no longer elicits a granulomatous reaction or affords antituberculosis immunity. We think it is a precursor or autolytic product of the polymeric peptidoglycolipid of the cell wall skeleton. We



TEXT-FIGURE 3.—Chromatogram (schematic) of the free lipids extracted from the tubercle bacillus. P_3 and P_4 are active lipids, toxic to mice, and apparently necessary for granuloma formation. Top material consists of low-molecular weight fragments of the cell wall skeleton (text-fig. 2).

are presently isolating by centrifugal chromatography the pure form of monomeric or oligomeric peptidoglycolipid.

RESULTS

Table 1 shows the results of a tumor-suppression experiment, in which untreated cell wall or the cell wall skeleton attached to oil droplets were injected intradermally (id), together with 10^6 ascites line-10 tumor cells, into strain-2 guinea pigs (*1*). Tumor growth was suppressed in 3 of 4 animals given 37.5 μg untreated cell walls. A similar dose of a cell wall skeleton preparation was completely effective; with as little as 18.75 μg cell wall skeleton, tumors grew in only 2 of 4 animals. In all controls, tumors grew progressively and killed the animals.

TABLE 1.—Suppression of tumor growth by Pasteur BCG cell walls attached to oil droplets

Fractions mixed with 10^6 tumor cells administered id	Dose (μg)	Results 5 weeks after injection
Cell walls	37.5	1/4*
Cell wall skeleton	37.5	0/4
	18.75	2/4
Tween-saline and oil droplets	—	4/4

* Number of animals with tumor/No. tested.

To demonstrate that systemic tumor immunity had been established in the animals that survived, they were challenged id after 10 weeks with 10^6 tumor cells contralateral to the side on which they were immunized. Animals in which tumor growth had been suppressed at the site of inoculation showed a cutaneous reaction to tumor cells and rejected the systemic tumor challenge (table 2).

TABLE 2.—Tumor immunity produced with cell walls attached to oil droplets

Preparation mixed with 10^6 tumor cells, administered id	Results of challenge with 10^6 tumor cells 10 weeks after immunization	
	Cutaneous reaction (24 hr after challenge)	Tumor incidence* (1 month after challenge)
Untreated cell walls	+++†	0/3‡
Cell wall skeleton	+++	0/4
Normal controls	0§	3/3

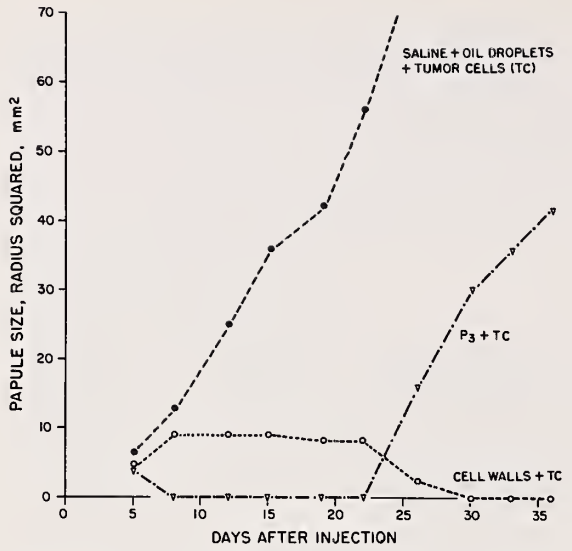
* At challenge site.
† Bright pink with pale ischemic center.
‡ Number of animals with tumors/No. tested.
§ Pale.

Table 3 summarizes results of 2 experiments showing that P_3, isolated from a cord factor sample by preparative centrifugal chromatography and attached to oil droplets, was not effective in suppressing tumor growth. P_3, cell walls, and viable BCG produced inflammatory reactions; however, the reactions given by P_3 disappeared much more quickly than those produced by cell walls, probably because this nitrogen-free fraction lacks the ability to induce delayed hypersensitivity to itself or to purified protein derivative (Meyer et al., unpublished data). P_3 injected together with tumor cells totally suppressed tumor growth initially (text-fig. 4). After 2–3 weeks, rapidly growing tumors appeared. We have reproduced this phenomenon, but the reason for it is still not clear. In vitro, at least, P_3

TABLE 3.—Suppression of tumor growth by mycobacterial fractions attached to oil droplets

Fractions mixed with 10^6 tumor cells, administered id	Dose (μg)	Results 5 weeks after inoculation of tumor cells
Untreated cell walls	75	1/5* (0/5)†
P_3	75	5/5 (5/5)
Tween-saline and oil droplets	—	5/5 5/5

* Number of animals with tumor/No. tested.
† Numbers in parentheses indicate results of a second parallel experiment.



TEXT-FIGURE 4.—Curves of tumor growth suppression by mycobacterial fractions attached to oil droplets. ● = control; ▼ = 75 γ P_3 in oil droplet/Tween-saline suspension + 10^6 tumor cells; ○ = 75 γ BCG cell walls on oil droplets + 10^6 tumor cells.

is not toxic to tumor cells (determined by trypan blue exclusion). The vigorous inflammatory response to id injected P_3 which occurs within the first 24 hours and which is stronger than that given by cell walls, is perhaps sufficient to slow down tumor growth by providing an environment not conducive to tumor cell proliferation.

Table 4 shows the results of a regression experiment in which the preparations attached to oil droplets were injected into preestablished, 8-day-old solid tumors. One tumor, inoculated with 300 μg P_3, regressed after a few days; a 300-μg dose of cell wall skeleton was not effective. However, when 150 μg each of P_3 and the cell wall skeleton were combined and injected, 3 of 5 tumors regressed, and growth of metastases was prevented. Subsequent challenge with 10^6 tumor cells revealed that systemic tumor immunity had been established.

TABLE 4.—Tumor regression produced by injecting mycobacterial preparations into established tumors. P_3 + cell wall skeleton was about as effective as the untreated cell wall in doses used here

Material injected into 8-day-old tumor	Dose (μg)	Results 30 days after treatment
P_3 (single-component toxin)	300	4/5* (5/5)†
	75	5/5
Cell wall skeleton	300	5/5
	75	5/5
P_3 + cell wall skeleton	150+150	2/5
	37.5+37.5	4/5
Untreated cell walls	300	2/5
	75	3/5
Tween-saline and oil droplets	—	5/5

* Number of animals with tumor/No. tested.
† Results from a parallel experiment.

DISCUSSION

The results of this regression-type experiment and other data not presented here suggest that toxic lipid is essential for destroying established tumors, but that it needs the help of an adjuvant and/or other functional material supplied by the cell wall skeleton. Possibly, as in the case of immunity to tuberculosis, the combination of toxin and cell wall skeleton is necessary to elicit a strong inflammatory granulomatous reaction. This is supported by the observation that injection of a nonfractionated mixture of free-lipid extract which contained P_3 and top material (mono- or oligomeric peptidoglycolipid) caused regression in at least 50% of established tumors (Ribi, Zbar et al., unpublished data). However, for the induction of systemic tumor immunity in our suppression-type test system, the adjuvant-active cell wall skeleton alone was sufficient when administered with tumor cells. If the soluble toxin was essential, only a minute quantity was required.

We have shown that, to cause regression of established line-10 tumors in strain-2 guinea pigs, we need the toxic lipid P_3, probably as a nonspecific inflammatory agent, as well as cell wall skeleton, which we suspect is an adjuvant for the induction of specific tumor immunity. A combination of P_3 and cell wall skeleton will produce intense granuloma (in mice), whereas neither will by itself. We have not shown unequivocally that tumor immunity is necessary for established tumor regression—only a constant association between the two. Conceivably, the nonspecific granulomatous inflammatory response alone is sufficient to destroy the tumor cells and the production of tumor immunity is a by-product of this reaction.

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DISCUSSION

D. W. Weiss: We will have questions specifically on Dr. Ribi's presentation and then go on to a discussion of questions pertaining to mechanism and to in vitro correlates.

R. L. Simmons: Haven't you and Dr. Zbar done some in vitro studies to study the mechanisms? Dr. Borsos mentioned at the Gordon Research Conference—and I think it's very pertinent here—in vitro studies on the mechanism of tumor destruction with BCG.

T. Borsos: That was in mice. We can get into this subject when we discuss the in vitro part which will come up later.

G. B. Mackaness: Did the animals given the P_3 develop tuberculin sensitivity?

E. Ribi: No. Neither does P_3 give delayed hypersensitivity to itself.

M. Chase: On second thought, I am inclined to congratulate Dr. Ribi on 2 points: 1) the effort he puts into his experiment; and 2) his lantern slides which are visible.

Ribi: Thank you, Dr. Chase.

R. B. Herberman: Dr. Ribi, can one conclude from your last table that both factors were needed, since there wasn't a complete dose-response curve constructed with each of the materials? For instance, the P_3 at the 300 μg gave some suggestion of protection. Perhaps P_3 150 μg gave the peak response.

Ribi: We are doing more complete dose-response assays. We also have to vary the proportions of the cell wall skeleton to the P_3.

F. M. Berger: Does the cell skeleton produce delayed hypersensitivity?

Ribi: Yes. I. Azuma estimated that the protein content is about 1.04%.

S. H. Stone: I thought it was pertinent to remark that J. Freund used to refer to his adjuvant in the plural, complete adjuvants, indicating that several activities are inherent in the adjuvant.

Weiss: We will continue with a short comment by Dr. Chedid, again on the questions of fractions of BCG. But I would like to suggest strongly that the speakers and the discussants remember that our common denominator in all of this discussion this afternoon is to concentrate on the question of whether animal models serve as models for human immunotherapy and to attempt, as far as possible, to relate (without too much philosophizing) our comments and data to the human situation.

L. Chedid: Several microorganisms have been reported to have strong immunostimulant activity. However, differences exist between organisms which are all good adjuvants, such as *Corynebacterium parvum* or *Bordetella pertussis*, and mycobacteria. The first 2 sensitize the host to histamine, whereas mycobacteria do not.

Mycobacteria are endowed with many biologic activities. Besides being immunogens and also capable of increasing the host's "nonspecific" resistance toward a large spectrum of bacteria, viruses, and tumors, they can equally exert undesirable properties, such as hyperreactivity to endotoxins, tuberculin sensitivity,

granuloma formation, lymphoid tissue hyperplasia, and allergic polyarthritis. Now, do all these actvities go hand in hand or can one eliminate the undesirable properties?

My aim today is to give you some data concerning a rather well-defined component, the water-soluble adjuvant (WSA), which can be obtained from cell walls or even from whole bacteria and which has been prepared by E. Lederer and his group.

WSA cannot render a mouse hyperreactive to the endotoxin, a phenomenon which may be important physiopathologically because of our gram-negative intestional flora. Moreover, WSA does not elicit tuberculin sensitivity and does not induce granuloma formation or allergic polyarthritis.

Weiss: Dr. Chedid, has this material been tested in a tumor immunity system?

Chedid: No.

Weiss: If it has not, perhaps we should leave this subject until later, because this meeting is not intended to include discussion on various tubercle bacillus materials with no relevance to tumor immunity. This would open it up to a much too-wide range. If there has been some work with this material on tumor immunity, then, of course, this subject would be pertinent.

Chedid: This material has not been tested against tumors. However, because many investigators have used BCG to immunize against tumors, I believe that you would have been interested in the data relating to the in vitro mechanism of this material, which is the active component of Freund's complete adjuvant.

Weiss: I am personally interested, but my instructions by the organizers are to focus this session entirely on tumor immunity models.

Chedid: In that case, I can only refer hastily to other non-water-soluble fractions prepared by Dr. Lederer which are either delipidated and enzyme-treated whole cells or delipidated and enzyme-treated cell walls. These preparations tested by us can protect mice against 2 tumor systems: 1) the Erhlich ascites carcinoma and 2) a syngeneic lymphoid leukemia, which has appeared sponstaneously in our laboratory in a hybrid of C57BL and AKR mice. These preparations, which have very strong antitimor activity, do not, however, sensitize mice to endotoxins, again showing that the different activities can be separated. However, I am sorry that I cannot show you the data.

Weiss: Is there any question pertaining to this insoluble material which Dr. Chedid mentioned in passing and which does have antitumor activity?

Ribi: Dr. Hiu recently published an article[1] describing a WSA and an antitumor factory (mycobacteria adjuvant and antitumor fraction); in our opinion, this is probably very much the same material as that described by Dr. Chedid.

M. Tishler: The methanol-extraction residue (MER) does have this identical effect that was reported by Hiu, as far as I can tell. The yield we got is quite a bit less than what Hiu reported he got from the French BCG. Hiu reported about a 10% yield. We could get about a 2.5% yield. Also, we obtained this material by a different method and obtained it in a way quite different from the way Hiu did.

R. Weiner: I have comparative data on the Hiu and MER material, again in our screening system in which we used tumors.

Tishler: Is that the water soluble form of the MER?

Weiner: I have both: the insoluble and soluble Hiu fractions from MER.

Weiss: Would you, first of all, tell the group what the "water-soluble Hiu material" is? Everybody may not be familiar with it.

Weiner: I am the wrong one to do this. Hiu-1 is an insoluble component of the BCG membrane. I'm not familiar enough with the chemical method involved in its production to report on it.

Tishler: The method is very simple. It is called "hydrogenolysis." He has a catalyst which promotes hydrogenation of the substance bringing about a cleavage of the molecule into water-soluble, presumably smaller-sized molecules. And this is the thing on which Hiu has reported and has given some physical and biologic data. As I said our physical data appear to be identical, or pretty close to being identical, with what Hiu reported.

Weiss: Would you want to summarize the biologic information you have on this material?

Weiner: I will do this tomorrow.

Weiss: Is there any other question pertaining to Dr. Chedid's remarks?

Tishler: You've got it on MER which had been subjected to hydrogenolysis?

Weiner: That is taking Hiu's material, given to us by Hiu, and comparing it with MER produced by Dr. Weiss' method.

Weiss: But produced by Hiu? It is not a batch that you received from us?

Weiner: No.

Weiss: We will continue our discussion now with questions pertaining to mechanism and to in vitro correlates. There will be considerable divergences and differences in the various subtopics within this category. May I first call on Dr. Waksman.

B. H. Waksman: I am really quite shocked at what has been going on. Both the Chairman and Dr. Borsos have in essence said that this subject is not scientific but simply empiric. We will test materials at various doses, compare what we can extract from this place or that place, and see what works; but we will not really address ourselves to questions of how they work.

I came to this meeting because the word "mechanism" appeared. When I said to Dr. Borsos that I don't work on tumors or on BCG, he said, "Come anyway. We need people who are concerned with mechanisms." I would be delighted to address you for a few minutes on the subject of mechanisms, but my slides don't concern BCG or tumors.

[1] Hiu IJ: Water-soluble and lipid-free fraction from BCG with adjuvant and antitumour activity. Nature [New Biol] (Lond) 238:241–242, 1972.

I am also a bit shocked that the group assembled here is willing to accept Dr. Borsos' dictum and address itself only to questions specifically involving tumor and BCG.

As you all know, digitalis—which was a folk medicine—works. And it worked before we knew anything about its mechanism. But there are many things we are never going to be able to make work unless we find out their mechanism of action. Evidence is ample in the conflicting data we have heard today that we are not dealing with a simple technique that is going to cure cancer. If we want to make sense out of what we're doing, we must understand the mechanisms we are stimulating.

Weiss: Speaking for myself and not for the other chairman or for Dr. Borsos, let me make my position entirely clear. A discussion of mechanism is vital!

I attempted to make a distinction by which I still stand. Our function is to discuss from a mechanistic as well as from a behavioral point of view the effects of BCG and BCG-related compounds in tumor immunotherapy. If we talk in general about the biologic activities of BCG or BCG fractions in many other systems, this certainly would be extremely interesting. But it is not—as I was informed and instructed—within the scope of this meeting.

Borsos: You have clearly stated my entire position. One purpose of this meeting was indeed to explore the mechanism of how BCG treatment can rid an animal of its cancer. I would very much like to hear a general discussion on the mechanism of how cellular or humoral immunity is stimulated by these various factors. But at this meeting we are addressing ourselves to the question of the use of BCG in the therapy of cancer. The mechanism of this process, for example, may have nothing to do with the delayed reaction of the type Dr. Chase or you describe. How does one know that, if one studies the mechanism of a nontumor model system, one would indeed study the mechanism of tumor rejection? Therefore, I believe the only relevant endpoint in which we are interested in these studies is the elimination of existing tumors, and every experimental design must take this fact into account. The decision to limit the scope of this meeting was mine; therefore, I am the one to be blamed. If I am wrong, I will stand corrected on this.

Chase: Has anybody today talked about the "how" of the action of BCG?

Borsos: Not yet. And this is what Dr. Weiss was just coming to.

Weiss: If I may add one more word to what Dr. Borsos just said, I am not myself prepared to say a priori what mechanism is or is not significant in terms of protection against tumors. Any discussion of mechanism of BCG action is valid with only the proviso that the speaker or the discussant attempts to relate mechanism to questions of tumor immunity. I take a very broad view of this, but attempts to relate discussion to tumor immunology must be made or the meeting will lose it cohesiveness. With this much of a comment, Dr. Waksman, would you be prepared to continue?

Waksman: I would, but I would like to hear the rest of Dr. Rosenthal's remarks and Dr. Chedid's remarks before I do.

Chedid: I believe the title of this meeting is "BCG and Cancer."

Borsos: No. "The Use of BCG in the Therapy of Cancer."

Chedid: Do you believe that it is not in the scope of this Conference to give data on how some components of BCG increase the cell-mediated immune response?

Borsos: I am very much interested in it. That's not the point. But how do you know that increasing cell immunity as measured by delayed reaction of nontumor antigens . . .

Weiss: I'm sorry. As a point of order, I think one can simplify this. Dr. Chedid, if you are discussing a fraction of BCG that has an effect on cellular immunity and if that fraction has also been shown to be in any way involved in a tumor system, discussion of that fraction is entirely pertinent. If you speak of fractions which have never been looked at in the tumor system, the discussion is not pertinent.

I do not put restrictions on questions of mechanism. We don't know what the mechanisms are and, therefore, anything is valid for discussion. But if we talk about the maerials that have never been assayed in a tumor system, then I think we're out of order.

Chedid: I understand your point of view; but objectively I really cannot share it.

Weiss: Dr. Waksman, do you wish to continue?

Waksman: Yes. But I should first comment on Dr. Borsos' last remark. The fact that you have never tested a phenomenon in a tumor doesn't mean it isn't relevant. It may be more relevant than all the ones that have been tested. In fact, one purpose of a meeting like this should be to bring to the attention of this group what are the phenomena they should be looking at in tumors not what did someone happen to think of a tumor tested in the past.

Borsos: We have decided the phenomenon we should look at ultimately is immunotherapy: rejection of tumor.

Waksman: Absolutely.

Borsos: And that is the mechanism we should study. That's exactly my point. And if your mechanism doesn't apply to that one point, it has no relevance to the scope of this Conference.

Waksman: Let me make my remarks, and then you will judge if this is relevant to immunotherapy.

As everybody here is aware, there are at last 3 distinct classes of cells which at one time or another damage tumors and which are, therefore, potentially meaningful in immunotherapy. Let me say a word about each of these.

First of all, we have the so-called T-lymphocytes, the thymus-processed lymphocytes. These can produce direct cytotoxic destruction of tumor cells, as shown in experiments like those of Brunner and his colleagues.

Borsos: May I interrupt you for one second? None of these experiments deals with immunotherapy. All are prevention models.

Weiss: But that does not invalidate their pertinency.

Borsos: I think we are coming to the crux of the

point. Dr. Terry and Dr. Rapp emphasized that we have to clearly distinguish between immunotherapy and prevention models.

Waksman: About half of the remarks today have been about prevention models and not about immunotherapy. And I think many of the mechanisms which apply in the one case will be found to apply in the other when they're adequately studied.

The next thing the T-cells do, besides exerting a direct cytotoxic effect, is to release soluble mediators which kill tumor cells. The term "lymphotoxin" is used as a general category for such materials. But I will remind you that additional names have been placed on other soluble products, such as proliferation-inhibitory factor, cloning-inhibition factor, inhibitor of DNA synthesis, etc. Until these substances are isolated, characterized, and studied in a number of systems, we shall not really know very much about them. But all of them are possibly what we are talking about today without knowing it.

B-cells have also been shown to kill target cells when they are coated with antibody. The B-lymphocyte is not processed in the thymus and is distinguishable by many criteria, e.g., a high concentration of surface immunoglobulin. There is now evidence that, in at least some situations of so-called tumor immunity, the mediating mechanism may be the formation of humoral antibody which coats the target cell, which is then killed by a B-lymphocyte activated by reacting with that coated cell. Here we have another distinct mechanism.

Then we have the macrophage, which can kill tumor cells by direct phagocytosis, as was shown years ago by B. Amos, especially when it is coated with specific cytophilic antibody. It can also kill by cell-to-cell contact, as shown by Dr. Hanna just a few minutes ago. In addition, it can release cytotoxic factors which have been described, on the one hand, as proteins and, on the other, as phospholipids. Very likely there are several cytotoxic factors released by macrophages. They are known to release lysosomal hydrolases into the ambient medium, and these can kill target cells.

Now I'm sure that's not a complete list, but it's the one that occurred to me as I was listening to the discussion.

The next important point is that these cells cooperate with each other—the so-called "helper" phenomenon in immunology. It's been known for about 5 years that T-cells cooperate with B-cells, and that anything which activates T-cells will help to turn on B-cells so that they can act. The usual test systems involve antibody formation. However, since we just heard that B-cells can kill tumor cells in an appropriate situation, anything that helps turn B-cells on is relevant to the subject of today's discussion.

In addition, T-cells can cooperate with T-cells. This has been shown clearly in a couple of systems and presumably applies to all T-cell activities including the killer function.

Then there is the cooperation of macrophages with T-cells, which is well recognized in many systems. In fact, this is one of the "classical" phenomena of immunology. Here at least 4 submechanisms have been described:

1) The macrophage can process antigen and render it more immunogenic. 2) A macrophage can supply an RNA or membrane constituent, which forms a "superantigen" in combination with the antigen or with antigen fragments. 3) The macrophage can actually "present" the antigen in some mechanical way to the T-cell. 4) The macrophage releases a so-called "lymphocyte-activating factor."

These mechanisms have all been validated by thorough experiments. We cannot say at this point which is important in tumor killing and which is not. If these mechanisms haven't been tested on a tumor up to this afternoon, that doesn't mean they may not be the precise mechanism in which we are most interested.

Another side of the coin, which has been recognized only in the last 2 years, is that these cell combinations have suppressor functions as well. Several people this morning referred rather casually to competition of antigens. It is now a well-recognized fact that, if one stimulates an immune response, a few days later the lymphoid organs in which this immune response occurs will actively suppress responses to another antigen. This is one of the types of competition mediated by a T-cell, for which the term "suppressor T-cell" has been coined.

Allotype suppression is also mediated by a suppressor T-cell. Immunosuppression in the graft-versus-host reaction appears to be similarly mediated by suppressor T cells. And evidence is accumulating that about half of what was formerly thought to be tolerance is not tolerance but is also mediated by suppressor T-cells.

I won't get into enhancement, which everybody thinks is mediated by antibody, but which very well may be mediated by antigen-antibody complexes stimulating suppressor T-cells. Thus the suppressor T-cell is an extraordinarily important mechanism in immunology, and this applies to immune responses to tumors, above all.

Now, we have recently uncovered—and this is what my slides were about, but I don't dare show them.

Weiss: Please do.

Waksman: No, because we have used phytohemagglutinin (PHA) and things which are very far from tumor cells. However, their relevance is absolutely clear, no matter what was used. We have shown that there are macrophage-lymphocyte combinations which are suppressors together where neither component by itself is a suppressor. This provides essentially a circular mechanism, because a stimulated T-cell can release substance(s) which stimulate or activate macrophages. The activated macrophage releases a lymphocyte-activating factor which stimulates T-cells, in turn, and so on, round and round.

So if you use any agent that turns on either the T-cell or the macrophage, you produce a constant crescendo effect, whatever the effect is. The effects we have measured have been suppressor effects on a variety of responses in vitro. Suppressor effects are what we are concerned with in tumor immunity or tumor therapy. We either want to kill the tumor or suppress its DNA synthesis. Also we are concerned with an immune response, and this suppressor mechanism is directed as well to immune responses.

Weiss: May I ask you to please show your slides. I think it is pertinent; if the Chairman has some prerogatives, I would use it to say this is entirely within the scope of the meeting.

Waksman: Thank you very much, Dr. Weiss, but I think it would take extra time, so let's not. I would rather make a couple of additional points.

BCG is one of the main topics under discussion today. BCG affects the type of systems that I speak of in two clear ways. We just heard a moment ago from Dr. Chedid that BCG stimulates macrophages. It also sensitizes and produces a population of reactive T-cells that will react to BCG antigens. In the cooperation between macrophage and T-cell, the evidence is now persuasive that what we refer to as adjuvant effect is mediated by this cell combination, by causing the macrophage to release lymphocyte-activating factor which helps to turn on the reactive T-cell. When we use a substance like BCG, we are very much concerned with precisely this kind of a cell unit in the lymphoid organs.

Two other workers, M. Scott—who is now at the Trudeau Institute—and O. Sjöberg—one of G. Möller's collaborators at the Karolinska Institutet—have studied the graft-versus-host reaction and *C. parvum,* both well-known adjuvants.

I know we are not totally hung up on BCG because Dr. Klein is on tomorrow's program, and certainly most of his interesting work until quite recently was with agents other than BCG. Dr. Klein was essentially seeking a technique for activation of helper T-cells in a system involving, presumably, other T-cells, or possibly a macrophage–T-cell combination.

The suppressor function we have described with PHA has been confirmed by Scott and by Sjöberg using *C. parvum* and the graft-versus-host reaction. Both these investigators have had great difficulty disentangling the macrophage from the T-cell in the mechanism, and so have we. But the evidence is unequivocal that there are 2 types of cells involved.

Now I'm sorry to have made such a fuss, but if we are going to talk about mechanism we cannot constantly be addressing ourselves only to tumors. Some of the most important things we know were found out first in other systems and may or may not be relevant to tumors, but that must be looked into.

M. G. Hanna, Jr.: I would like to take up where Dr. Waksman ended his comments, particularly with respect to the aspect of T-cell cooperation with cells of the macrophage-histiocyte compartment and the role of this type of cell cooperation in the immunotherapy model incorporating a syngeneic transplantable hepatocarcinoma in inbred guinea pigs. Many of the things relating to mechanism have been said. However, possibly they have not been put in the right order.

In terms of the guinea pig—BCG therapy model, where a single intralesional injection can cause a BCG-histiocytosis which can result in regression of the tumor as well as elimination of regional metastases, it was important that we consider this as a nonspecific histiocytic reaction, capable of killing tumor cells.

Along these lines, it was then important to determine whether other types of nonspecific inflammatory reactions or nonspecific lymphoproliferative responses, such as those produced during the development of delayed-type hypersensitivity, could also cause tumor regression and eliminate lymph node metastases. The latter was an important issue, since, in the treatment group where tumor excision was performed in place of BCG therapy, a conventional lymphoproliferative response of the regional lymph nodes in the absence of histiocytosis was insufficient to inhibit growth of metastatic cells. Therefore, we performed studies to compare the effectiveness of BCG and other agents in promoting tumor regression and eliminating regional metastases. Turpentine was intralesionally injected, since it produces an acute inflammatory reaction in the skin and provides a means for evaluating the contribution of the immediate inflammatory reaction to tumor regression. Oxazolone, known to produce classic delayed-type hypersensitivity reactions in guinea pigs, was used as a sensitizing agent, topically applied to tumors of one group of test animals. Vaccinia virus was also intralesionally injected in a separate group and was used because, clinically, intralesional injection of this virus can produce regression of metastatic malignant melanoma.

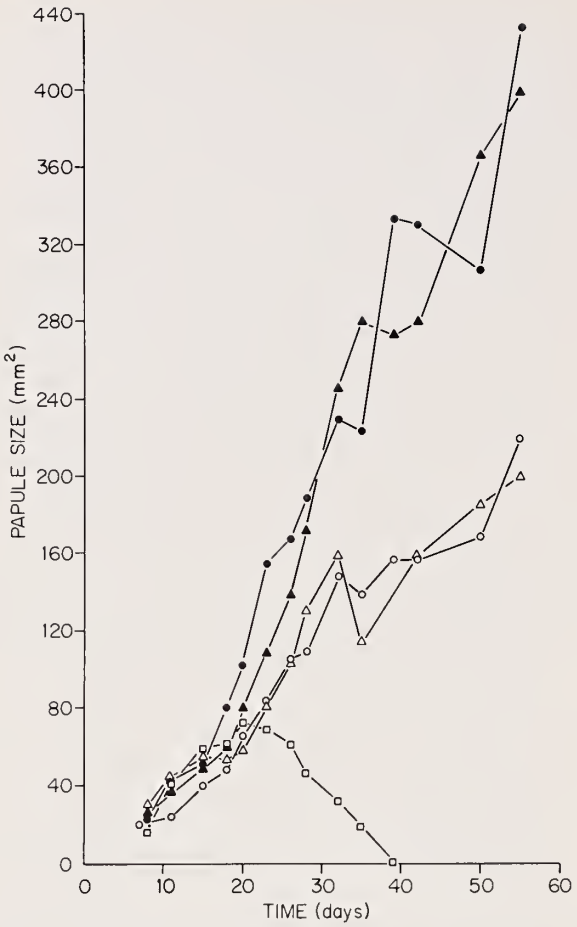
The results of these studies were remarkably clear (text-fig. 1). Cellular reactions in the regional lymph node, which were characteristic of the development of delayed hypersensitivity as produced by vaccinia virus and oxazolone, had no detrimental effects on the tumors and did not alter metastatic growth. The turpentine-induced inflammatory reaction at the tumor site also did not suppress tumor growth. Both turpentine and oxazolone treatments, however, enhanced tumor growth in the skin.

The results also demonstrated that intradermal tumors completely regressed after treatment with BCG and that regional lymph node metastases were eliminated. The characteristic morphology was a BCG-mediated granulomatous reaction in both the tumor site and the regional lymph nodes, as I described earlier this afternoon. As I also described, both histologically and ultrastructurally, histiocytes appeared to be the major effector cells in this reaction.

This is not the whole story, however, when we think about mechanism. Dr. Zbar mentioned this morning that the BCG-mediated regression of the transplantable syngeneic hepatocarcinoma and the elimination of regional lymph node metastases requires 2 steps. The first step, as described by Dr. Zbar, is immunologically specific for BCG antigens. We also have data to support this analysis, based on the inability of BCG to regress tumors in animals made immunologically incompetent by anti-lymphocyte serum.

The second step, effective in destroying tumor cells, is probably immunologically nonspecific, and this is BCG histiocytosis. Therefore, during the early stages of the BCG reaction after intralesional injection, one has to consider seriously a T-cell or lymphocyte cooperation with cells of the macrophage-histiocyte series.

It is generally accepted that cells of the macrophage-



TEXT-FIGURE 1.—Tumor growth calculated from mean papule size (r^2) after various treatments: ○, saline (controls); □, BCG; △, vaccinia virus; ●, oxazolone; and ▲, turpentine.

histiocyte series are the major effector cells in nonprogressing tuberculous lesions of the skin, and/or draining lymph nodes, after intradermal BCG injection. Some of these cells ingest and destroy the granulomagenic organisms, as seen in figure 1. This produces a number of morphologic and functional alterations (activation); e.g., mesenchymal cells, as well as their daughter cells, assume the characteristics of primitive epithelioid cells. Ando et al.[2] demonstrated that lymphocytes are required in this response. It has been shown that delayed hypersensitivity enhances the number of mononuclear effector cells infiltrating the lesion and undergoing activation and division. In guinea pigs, the overall cellular activity results in this granulomatous reaction at the site of BCG localization. Therefore, lymphocytes, possibly T-cells, have a specific role in the control of mononuclear cell infiltration into the tuberculous reaction site.

A further point that has to be evaluated is evidence that, during the course of tumor regression, specific tumor immunity is developed, mediated by lymphocytes as well as by detectable antitumor specific antibody. It has been demonstrated that the syngeneic hepatocarcinomas used in the present study are rejected when they are reintroduced into guinea pigs that have recovered from tumor cell-BCG granulomatous reactivity. These results suggest that lymphocyte sensitization occurs during the granulomatous reaction; based on studies demonstrating that regional lymph nodes draining BCG sensitization sites are anergic, one would have to assume that the developing lymphocyte sensitization during a BCG granulomatous response probably occurs systemically in peripheral lymphatic tissue.

Therefore, I think we have at least 2 steps where lymphocyte or T-cell involvement is important: 1) in the initial cooperation with cells of the macrophage-histiocyte series in establishment of the BCG histiocytosis and 2) the important aspect to the therapy model, with respect to elimination of distant metastasis that might occur, in the eventual sensitization of lymphocytes resulting in specific tumor immunity that exists in these guinea pigs after the tumor has regressed and regional lymph node metastasis has been eliminated by the BCG histiocytosis.[2]

Waksman: May I add one footnote to Dr. Hanna's remarks. It looks as though the very same cell system can give you either help or suppression depending on timing and dose. The fact, therefore, that you could not show suppression by activating T-cells in the local node does not necessarily mean that that is not the operating mechanism.

Hanna: I agree. All it says is that the nonspecific T-cell proliferation response is not, in itself, detrimental to the tumor growth. The point I am trying to make, however, is that T-cell cooperation with cells of the histiocyte-macrophage series is a requirement of the tuberculous reaction and that effective cell-mediated immunity involving T-cells is the ultimate immunologic achievement in these animals, resulting from BCG histiocytosis and its ability to eliminate tumor and metastases.

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[2] ANDO M, DANNENBERG AM, SHIMA K: Macrophage accumulation, division, maturation and digestive and microbicidal capacities in tuberculous lessons. II. Rate at which monoluclear cells enter and divide in primary BCG lesions and those of reinfection. J Immunol 109:8–19, 1972.

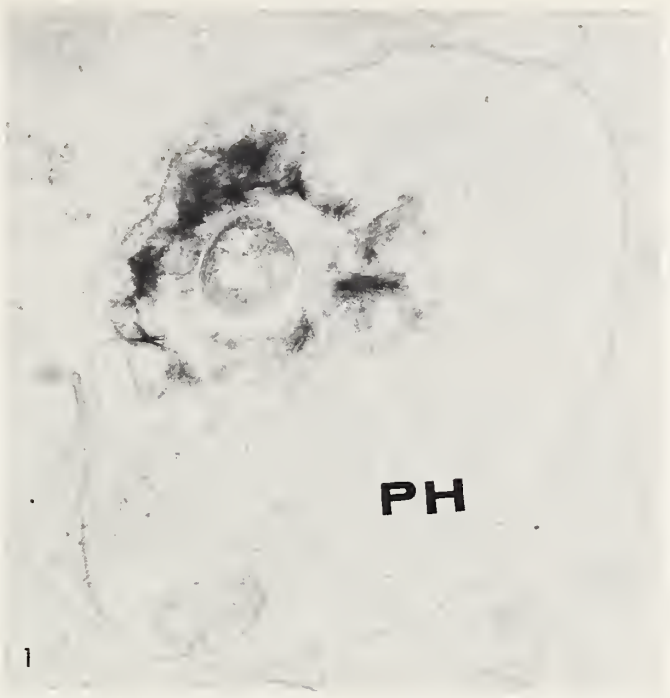


FIGURE 1.—A cross-sectioned bacillus is shown within a phagosome (PH) of a histiocyte in the lymph node draining a treated tumor at 4 days after the injection of BCG. The bacillus is relatively intact but is encompassed by a dark, polymorphous, crystalline-appearing material. × 45,000

Activated Macrophages and the Antitumor Action of BCG[1,2]

Peter Alexander, *Chester Beatty Research Institute, Sutton-Surrey, England*

SUMMARY—BCG influences tumor growth through various mechanisms and it is essential that these pathways be considered separately. Experiments are described which show that BCG, like other antigens, can cause the appearance in vivo of activated macrophages which kill tumor cells in an immunologically nonspecific way. The transition of immune macrophages from arming to activation is discussed and it is shown that malignant cells are killed in vivo as well as in vitro when a soluble derivitive from tubercle bacilli, PPD, is administered to mice that have been previously sensitized to BCG.—Natl Cancer Inst Monogr 39: 127–133, 1973.

Multiple Mechanisms by Which BCG Influences Tumor Growth

After injection of BCG into the host, there are local and systemic tissue changes which can, if the conditions are suitably manipulated, influence the growth of tumors. Also, BCG organisms have chemical constituents which are adjuvants and can enhance the immune reactions of the host to unrelated antigens. Experiments during the last 15 years have shown that various mechanisms are responsible for the antitumor actions of BCG; they depend on the conditions in which BCG is used. We can learn what value BCG may have for the control of tumors in man only by separating the different processes; little is gained by referring to "the" action of BCG or by considering it apart from other reticuloendothelial stimulants such as *Corynebacterium parvum*.

Among the reactions involved in the antitumor action of BCG are:

1) *Hyperplasia of the reticuloendothelial system* associated with greatly increased numbers of macrophages in liver, spleen, and lungs (*1*). Animals stimulated in this way have a greatly increased capacity to reject foreign microorganisms and, to a lesser extent, grafted tumor cells (*2*). This increased resistance persists several weeks after the injection of BCG (*3*), but the increased number of macrophages in such animals are not cytotoxic to tumor cells throughout the period in which protection can be observed (*4*). An explanation (*5*) for the increased resistance of BCG-treated animals is that host reaction against tumor requires both lymphoid cells and macrophages and that, under some situations, the availability of macrophages is limiting. The increase in total macrophages after nonspecific stimulation enables the lymphoid component of the immune response to exert its maximum activity. Systemic administration of BCG can also cause a small therapeutic effect, if BCG is given soon after the injection of tumor cells rather than as a prophylactic measure before (*6*). This therapeutic action is weak

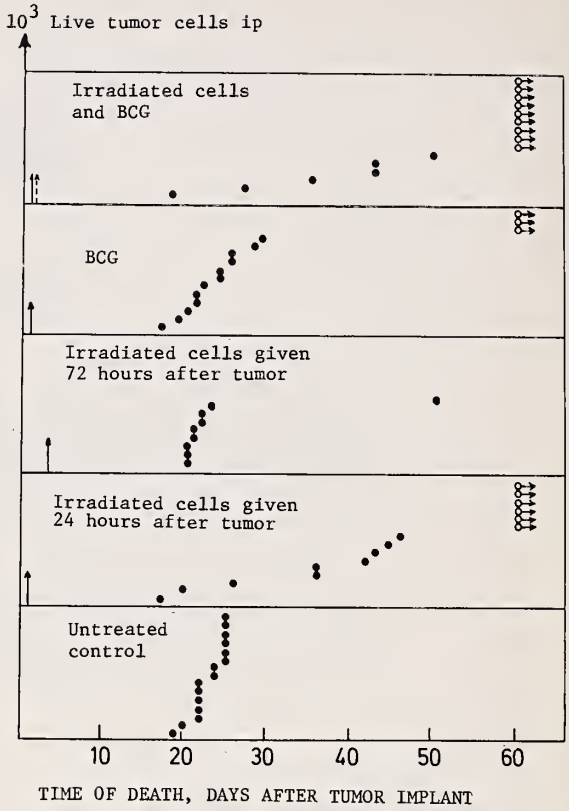
[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Supported by grants from the Medical Research Council and the Cancer Research Campaign.

and is seen in only a few experimental situations (*7*), and then only when the tumor load to be rejected is very small. It should not be confused with the antitumor action of intralesional injection of BCG.

2) *Localized tissue changes* in response to direct injection of BCG into the tumor. This effect is not confined to BCG, and Gorer and Boyse (*8*) noted in 1959 that tumors sometimes regress when infected with bacteria. Intradermally or subcutaneously (sc) injected BCG changes the local environment, making it unfavorable for the continuous tumor growth. This process has been described as "floating the tumour away in a flood of pus." This is probably an oversimplification—tumors occasionally persist for some time after infection, and direct action on the tumor cells by nonspecifically activated macrophages may be involved. The claim that this is therapy for metastatic cancer, because tumor cells in draining nodes are also killed, may be misleading. BCG organisms injected into the tumor necessarily gain access to the draining node where they will produce exactly the same local changes (*e.g.,* granuloma formation) as they do at the actual site of the injection. I believe it is wrong to imply that an effect occurring in draining nodes is a systemic manifestation after local treatment.

3) *Augmenting the antitumor effect* produced by administration of irradiated tumor cells. It is known that the growth of small amounts of tumors can be retarded or they can be made to regress totally by the injection at multiple sites of killed tumor cells (*7, 9, 10*). The mechanism probably involves stimulation of noninvolved nodes, which thereby increases the total immune reactivity of the host. In several situations, the administration of BCG with irradiated tumor cells is more therapeutic than the administration of irradiated tumor cells alone (*7, 10*). As already mentioned, systemic injection of BCG has only a feeble antitumor effect. This effect of BCG increasing the immunotherapeutic effect obtained with irradiated cells is illustrated in text-figure 1. That is not, however, a general phenomenon as shown in text-figure 2, where a tumor responds well to irradiated cells alone and the addition of BCG makes no difference. Whether this co-operative effect of BCG is due

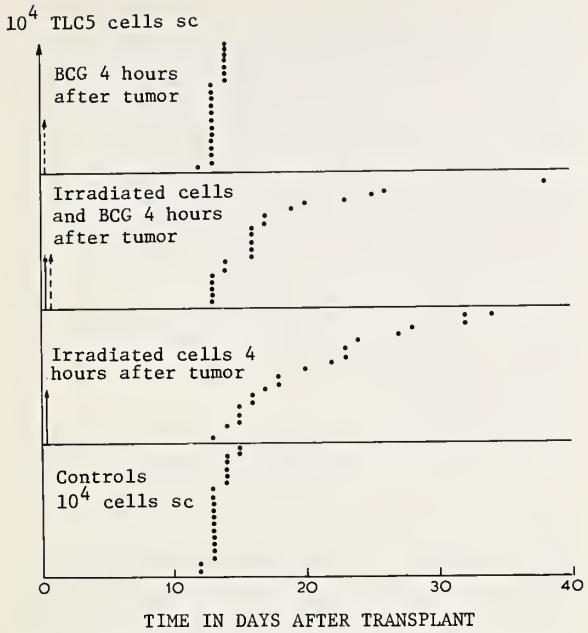


TEXT-FIGURE 1.—Treatment of L5178Y lymphoma with irradiated syngeneic tumor cells and BCG. 10^3 live L5178Y cells were injected intraperitoneally (ip) on day 0. 4×10^7 L5178Y cells (irradiated with 3000 R in vitro) were given ip as therapy. BCG, 0.6 mg of wet weight, was given ip. ● indicates day of death (*7*).

to its action as an adjuvant (i.e., it increases the specific immune response of the host to the injected irradiated tumor cells) or to the general stimulation of the reticuloendothelial system (the mechanism discussed above) is not yet clear.

BCG used in combination with active immunotherapy (e.g., irradiated tumor cells) seems to me the most promising clinical application for BCG because it affects disseminated disease. Some hopeful results have already been seen in treatment of acute leukemia (*see* papers by J. E. Sokal et al., G Mathé et al., and by R. Powles in this volume).

4) *Induction of activated macrophage.* BCG, like many other antigens, can cause the appearance in vivo of activated macrophages (*4, 11*), which kill tumor cells in an immunologically



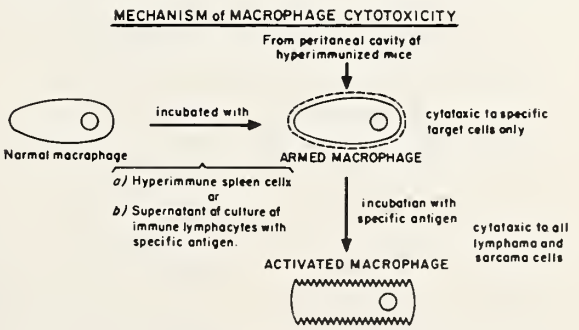
TEXT-FIGURE. 2.—Treatment of TLC5 lymphoma (CBA strain), injected sc. 5×10^7 irradiated TLC5 cells and BCG were given sc at multiple sites 4 hours later. ● indicates day of death (7).

nonspecific way. I will address this last type of BCG activity in the rest of this paper. As I already mentioned, such activated macrophages may play a part in the regression of skin tumors after local injection of BCG into the tumor (as described in this volume for guinea pig tumors and for human melanoma). Detailed in vitro and in vivo studies reported below suggest that activated macrophages cause the regression of skin tumors after the induction of a local delayed-hypersensitivity reaction, whether this is produced by BCG or by the application of a sensitizing antigen such as purified protein derivative (PPD) or dinitrochlorobenzene (DNCB) into a previously immunized host. E. Klein found this treatment valuable against basal cell carcinomas in man, as detailed by him in this volume.

Antitumor Action of Activated Macrophages

Macrophages which inhibit the growth of tumor cells in an immunologically specific way in vitro can be obtained in 3 ways: 1) directly from the peritoneal cavity of hyperimmunized mice (5); 2) by incubation of nonimmune macrophages with spleen cells from hyperimmune mice (*5, 12*); and 3) by treatment of nonimmune macrophages with a factor called "specific macrophage-arming factor" (SMAF), which is produced by immune T-cells when they are incubated for some hours with the specific antigen (*13–15*). The growth-inhibitory action of such macrophages can be directed against the normal transplantation antigen of the tumor cells (*16*) or, in syngeneic systems, against the tumor-specific antigens (*5, 12*). These immune or armed macrophages act by membrane contact with the target cell. Phagocytosis occurs late—only after the target cell has been killed (*5, 12*).

Immune or armed macrophages, after incubation with the specific antigen, undergo a change which we have called "activation," after which they can inhibit in vitro the growth of all the sarcoma and lymphoma cells we tested (*4*). This transition from arming to activation is diagrammed in text-figure 3. Table 1 outlines this transition, with data on 2 antigenically unrelated lymphoma cells—SL2 and TLX9. Macrophages from mice immunized against SL2 only affect the growth of SL2 cells, and the same is true of TLX9 cells. However, when anti-SL2 macrophages are incubated with SLS cells, they acquire the capacity to kill TLX9 cells as well, and the reverse applies to the TLX9. This transition to the activated state, however, requires the specific antigen, since anti-SL2 macrophages incubated with TLX do not become activated and will kill only SL2 cells. More relevant here is the effect of macrophages from mice immunized with BCG. Table 1 shows that macrophages taken from mice given injections of BCG



TEXT-FIGURE 3.—Transition of macrophages from armed to activated state (*4*).

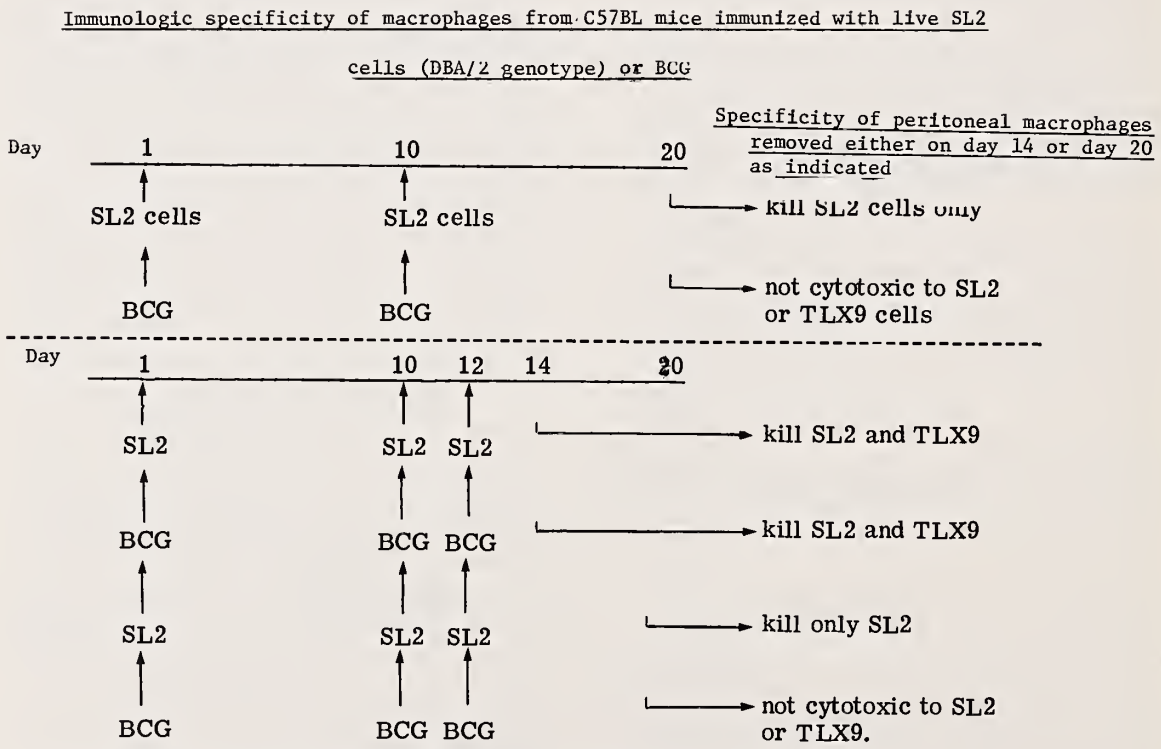
TABLE 1.—Cytotoxicity of macrophages from immunized mice*

CBA mice immunized against	Macrophages incubated with	Cytotoxicity to	
		SL2 cells	TLX9 cells
SL2 lymphoma (DBA/2)	No antigen	99%	<10%
TLX9 lymphoma (C57BL)	"	<10	99
BCG	"	<10	≤10
SL2 lymphoma	SL2 cells	99	79
	TLX9 cells	99	<10
	PPD	99	<10
TLX9 lymphoma	TLX9 cells	75	99
	SL2 cells	<10	99
	PPD	<10	99
BCG	"	92	84
	SL2 cells	<10	<10
	TLX9 cells	<10	<10

* Values from (*4*).

2 weeks earlier are not cytotoxic to tumor cells. However, if these macrophages are incubated for 4 hours with PPD, the soluble antigen from tubercle organisms, they then can kill both SL2 and TLX9 cells. Exactly the same phenomenon can be obtained with sheep red blood cells (*17*). The macrophages from mice immunized with sheep red blood cells are not at all cytotoxic to tumor cells. However, after a short period of incubation with sheep red blood cells, they become activated and are able to kill both lymphoma and sarcoma cells in an immunologically nonspecific way. The data in table 1 refer to the transition from arming to activation by contact with specific antigen of macrophages from immunized mice. The same phenomenon occurs if the macrophages are armed by contact with immune spleen cells (*4*) or by treatment with SMAF (*17*).

Transformation from an armed to an activated state by contact with specific antigen can also occur in vivo (*4, 11*). Text-figure 4 shows that, when mice are immunized 3 times with SL2 cells and the macrophages are taken 10 days after the last immunization, they are specifically cytotoxic; i.e., they affect SL2 cells but not TXL9 cells. However, if they are taken 1–3 days after the last injection of SL2 cells, they are activated and inhibit the growth of both SL2 and TLX9 cells. Presumably, macrophages were armed after



TEXT-FIGURE 4.—An example of macrophages with specific cytotoxicity (*11*).

2 immunizations and the final injection of SL2 cells caused them to become activated in vivo; lacking further antigen, they lost activation. The same pattern is seen with BCG. If the macrophages are taken > 10 days after the last BCG injection, they have no effect on tumor cells. However, if taken within 3 days of the last injection of BCG, they are activated and inhibit the growth of lymphoma and sarcoma cells in vitro. In these experiments the mice were injected with BCG manufactured by Glaxo. This particular strain does not persist for long in mice and it is likely that other BCG preparations which cause long-persisting infection may result in peritoneal macrophages remaining in an activated state for longer periods.

The phenomenon of activating macrophages in mice immunized against an antigen totally unrelated to tumors, such as BCG or sheep red blood cells, can be exploited for immunotherapy. This is illustrated (*18*) in a series of experiments summarized in table 2. Mice were pretreated with BCG and then 12 days later were challenged ip with lymphoma cells. The lymphoma cells grew almost as well in the BCG-pretreated as in normal mice (i.e., the protective action of nonspecific stimulation of the reticuloendothelial system is minimal in this situation). However, PPD injected 24 hours after ip inoculation of the tumor cells produced a marked antitumor effect in the BCG-pretreated mice. We attribute this antitumor action to the activation of peritoneal macrophages armed against BCG. However, this effect is strictly local. If the PPD is injected intravenously (iv) or sc, there is only a small effect on the ip tumor. Also, if the tumor is grown sc, ip injection of PPD has no effect. It is, however, possible to act with PPD given iv against a tumor challenge given iv into mice sensitized to BCG.

I believe this is another example of the phenomenon (described by E. Klein for man) where some skin tumors regress after painting with or injection of an antigen to which the patient has been sensitized (e.g., DNCB painting in DNCB-sensitized subjects or PPD injection into tumors of tuberculin-sensitive subjects). It must be stressed that the practical usefulness of all such treatments is limited because macrophage activation is largely confined to the site (or the node draining the site), where the antigen is injected. Hence, this procedure, as E. Klein has found, can be used only to eradicate accessible tumors; it does not get to the heart of the cancer problem—the elimination of distant metastases which cannot be treated by surgery or radiotherapy.

One can obtain activated macrophages from a persisting infection without a second antigenic challenge. The infection causes macrophages to be armed and the persistence of the infective agent ensures that the immune macrophages become and remain activated. This phenomenon was clearly demonstrated by the group of Remington (*19*) who found that, after infection with *Toxoplasma,* peritoneal macrophages of mice with active toxoplasmosis were cytotoxic to tumor cells in vitro (i.e., these macrophages were activated). These workers also showed that mice with macrophages activated by toxoplasmosis were more resistant to challenge with tumor cells (*20*) and to viral carcinogenesis (*21*). We made the same observation by accident: Our mouse colony became infected with *Pasteurella,* which persisted in the mice for

TABLE 2.—Effect of PPD on growth of lymphoma cells (L5178Y) in DBA/2 mice immunized with BCG*

Treatment protocol				Percent of mice surviving after injection of lymphoma cells		
Day 1	Day 8	Day 20	Day 21	<25 days	25–50 days	>50 days
Nil	Nil	10^4 L5178Y cells ip	Nil	90	10	0
"	"	"	PPD ip	100	0	0
BCG	BCG	"	Nil	60	40	0
"	"	"	PPD ip	30	20	50
"	"	"	PPD iv	60	40	0
"	"	"	PPD sc	40	60	0

* Unpublished data.

months; the macrophages from such mice were nonspecifically cytotoxic (*4*) and the mice were somewhat more resistant to tumor challenge (unpublished observation).

After local injection of BCG into tumors, the organisms persist for long periods in the tumor and in the nodes draining it (*see* M. G. Hanna, Jr., et al., in this volume). In this situation, there will be local activation of macrophages. While such activated macrophages probably contribute to the antitumor action of intralesional injection of BCG, as described for melanomas and for guinea pig tumors in this volume, I believe other factors may also come into play, since local BCG causes a very intense and long-persisting granulomatosis which differs from the delayed-type hypersensitivity reactions in which activated macrophages are produced. It seems important to establish whether these unpleasant and painful lesions are necessary for the effect seen in melanoma and in guinea pig tumors.

Selectivity of Activated Macrophages for Malignant Cells

While we found that activated macrophages, whether produced by specific interaction with antigen or by treatment with double-stranded RNA or endotoxin (*22*), inhibited the growth in vitro of lymphoma and sarcoma cells, we noted that they had no effect on the growth of rapidly dividing embryo fibroblasts (unpublished data). We did not test other rapidly dividing, nonmalignant cell lines to see if activated macrophages have the capacity to distinguish all normal cell lines from malignant cells. Remington's group reported that macrophages from mice infected with *Toxoplasma* inhibited the growth of the long-established line of mouse fibroblasts (L-cells), which can be considered to be malignant cells, but had no effect on the growth of normal mouse embryo cells (*19*). Subsequently, they showed that macrophages acted only against cells with abnormal growth behavior (*23, 24*). The fact that growing normal cells are not susceptible to growth inhibition by activated macrophages may help to explain why activated macrophages are apparently effective in vivo against some tumors.

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DISCUSSION

M. Chase: We heard a rather remarkable paper from Dr. Alexander in which he reported applying BCG on 2 or 3 occasions and purified protein derivative (PPD) on 1 occasion. Did any other speaker today report on multiple injections of BCG? That is a rather crucial point.

R. W. Baldwin: We give multiple injections.

Unidentified speaker: We give multiple injections also.

Chaser: Is there a superiority in your method?

Unidentified speaker: We found multiple injections were better than single.

M. R. Mardiney, Jr.: Dr. Alexander, can I interpret from your data that you believe that the macrophage has the innate ability to recognize nonself?

P. Alexander: I regret to say that the information on this is rather limited. In our experiments we have compared only lymphoma and sarcoma cells with the embryo fibroblast cells, and the macrophage killed the lymphoma and sarcoma cells but not the embryo fibroblast.

J. Remington has done the experiment more elegantly. He took embryo fibroblasts and cultured them. He found that the activated macrophage began to kill these cells as soon as they showed lack of contact inhibition. There was a very good correlation there.

But we can't say that this is an absolutely clear recognition of a malignant transformation because of sheep red blood cells—just a few papers have appeared on the lysis of sheep red blood cells, both in a specific way, by specifically immunizing animals as in the tumor cells and in an activated sense. But we can say lymphoma, sarcoma, and red blood cells are killed by activated macrophages; embryo fibroblasts are not.

Mardiney: Are these embryonal cells syngeneic?

Alexander: Yes.

R. E. Falk: O. A. Holtermann at Roswell Park Memorial Institute found that the macrophage will recognize the neoplastic cell vis-à-vis the embryonic cell or normal fibroblasts of the same species and will kill the tumor cells in the syngeneic system. But the macrophage will not touch another strain, malignant or not. So it then loses its determinant.

S. R. Rosenthal: I wanted to stress the differences between immunizing with a live BCG and a dead one. Dr. Alexander, you spoke about taking the lymphocytes from BCG-immunized animals and macrophage. It has been shown in other models that only the live BCG will have this effect. Do you have the same effect with the dead BCG?

Alexander: We haven't done this experiment with dead BCG but we've done it with ovalbumin. If you immunize animals with ovalbumin, take the macrophages 2 weeks later, and then add ovalbumin to these macrophages, the macrophages become activated and will kill tumor cells.

Rosenthal: It has been shown that lymphocytes from dead BCG-immunized animals added to macrophages will not be cytotoxic to the tubercle bacilli but lymphocytes from live BCG-immunized animals will.

Alexander: But one is using different endpoints. Incidentally, there's a man at Stanford University, who has done the same experiment with sheep red blood cells, in mice, too.

G. L. Bartlett: We have performed some experiments which were stimulated by Dr. Alexander's presentation here last spring. Instead of using peritoneal cells, we used spleen cells from BCG-immunized mice. We found that such cells were directly toxic to a variety of syngeneic tumor cells in vitro, if the culture medium contained PPD; there was no toxicity without PPD. PPD alone had no effect and nonimmune spleen cells had no effect. The PPD-stimulated spleen cells did not kill normal cells of 2 origins: mixed syngeneic embryonic cells or fibroblasts derived from explants of adult pleura.

We have subsequently found that the activity is—at least in part—present in the form of a soluble material in the supernatant of cultures of BCG-immune lymphocytes combined with PPD. The supernatants have the same differential toxicity for syngeneic tumor cells and normal cells.

B. Zbar: I just want to make a comment that links the problem of route of administration and mechanism. Most of the results from papers that have been reported in the rat by Dr. Baldwin, in the guinea pig in our laboratory, and in the mouse also in our laboratory all seem to

agree that, in order to get an optimal therapeutic effect, the bacteria and the tumor cell must mingle. The mechanism to which Dr. Alexander is referring and the experiments he showed in vivo also emphasize the requisite of having an immune reaction to antigens of this bacteria at the tumor site to get a therapeutic effect. That might be one thing on which some people could agree.

R. T. Prehn: I'd like to ask both Dr. Bartlett and Dr. Alexander this question: It's very intriguing that the macrophage can distinguish between tumor and normal embryo tissue, etc. It seems to me there have been studies suggesting that a cell in an in vitro test in suspension may be more susceptible to immune damage than one that's attached to a substrate. You mentioned that the macrophages become active when contact inhibition begins to be lost. Is it possible that the difference between the embryo cells and the tumor cells is not that one is embryo and one is tumor, but that one is better attached to the substrate than the other?

Alexander: That can't be the whole explanation because both the specific and the nonspecific macrophage killing is exerted very well and most easily studied in lymphoma cells which are free in suspension. But I would certainly guard against emphasizing that this may be a critical test of malignancy versus nonmalignancy.

I tried to emphasize that, in the sheep, the red blood cells are equally viable. And the number of normal tissues studied has still been very small. I might point out why this is so; although we've all referred to these macrophages in shorthand terms as being cytotoxic, actually in the syngeneic system—in the allogeneic system they do produce lysis—they are very cytostatic. Therefore, you can assay them only against dividing cells, and thus one is limited to embryo fibroblasts.

I would guess that some endothelial cells are quite sensitive to this, because we know macrophages treated with endotoxin also acquire this same degree of cytostasis and cytolysis in the allogeneic system. So I believe that some of the vascular damage produced by endotoxin may be due to damage of endothelial cells.

M. G. Hanna, Jr.: Dr. Alexander, could you give us a little more information about your observation that the histiocyte–tumor cell interaction seems to be a cell-surface contact phenomenon, and that later there appears to be phagocytosis of these tumor cells. I am interested in this because we performed an experiment using the guinea-pig therapy model on isolated histiocytes of regional lymph nodes draining sites of tumors that had regressed as a result of BCG intralesional injection. We mixed these histiocytes in vitro with syngeneic tumor cells and compared the effect of the regional lymph node histiocytes with peritoneal macrophages of the same animal. The histiocytes appeared to attach to the tumor cell surface and move along the cells in a sluglike manner, while the peritoneal macrophages of the same animal were actively engaged in phagocytosis of the tumor cells. There was really quite a marked difference in the approach of the 2 cells to the tumor cells.

Alexander: My guess would be—and we made a similar observation when we prepared our macrophages from spleen cells rather than from peritoneal cells—that the immunologic event is probably cell-to-cell contact killing. Some macrophages that are particularly well adapted to phagocytosis will then very regularly phagocytize; whereas other macrophages may not so readily phagocytize. As you well know, the histiocytes in extracellular fluid, of course, phagocytize very poorly. When people say macrophages are defined by their ability to phagocytize, they are quite wrong, because if one collects macrophages out of the afferent lymph . . .

Hanna: That's why I have avoided classifying the effector cells in the BCG tuberculous reaction as macrophages, because this denoted a cell actively phagocytizing; I do not see this with the effector cells of tuberculous reaction. I consider it wiser at the present time to simply call them "histiocytes."

Alexander: That phagocytosis is a later event is very easily shown with the lymphoma cells because you can actually, after 24 hours of contact, remove 90% of the cells, and you can show that they've undergone an irreversible change because they finally die when removed from the macrophages. But they are dead or they have lost reproductive capacity.

Hanna: Possibly what we see early in the response is the cell contact phenomenon which causes some kind of injury on the cell surface; then ultimately the cells undergo stages of degeneration, in which they are in a state rendering them particularly accessible to phagocytosis by macrophages.

Alexander: There are obviously 2 stages of recognition. When the cell has finally been severely damaged, then the reticuloendothelial system—just as it sees aged red blood cells—sees these and eats them.

Hanna: You say "recognition." Is that really recognition?

Alexander: Oh, I don't know. But we know what we mean by this.

Hanna: Yes.

J. E. Sokal: Dr. Zbar called for volunteers to disagree with him, so I will, at least partially. I am not sure that we always have to have contact between the BCG organism and the tumor cell. We should specify a little more clearly what precisely we're talking about. For a certain kind of immunization, obviously you do want contact, and you mix the BCG with the tumor.

However, in at least some kinds of immunotherapy with BCG, various investigators have shown unequivocal prolongation of survival by treating animals with BCG, independent of where the tumor is. For example, in protecting an animal against developing leukemia at some later time, obviously we are not putting the BCG into contact with any particular tumor cells. So certainly for the nonspecific effects of BCG, you don't need contact with tumor.

To get to Dr. Alexander's model, Dr. Klein has shown in man that induction of a nonspecific immunologic response in vivo does in fact produce this effect. So it is not just an in vitro system.

We have also done a similar kind of study with different antigens in a patient with reticulum cell sarcoma

and accessible lesions, who happened to be highly sensitive to a number of antigens. If one injects PPD or Varidase near a nodule, the nodule will disappear and the normal skin cells will not be affected.

Unidentified speaker: I wanted clarification on the point about persistence of BCG organisms after injection in the animal. Somebody said a while ago that the organisms disappeared, but I thought they persisted live for a long time.

Rosenthal: This is the great difference between using a live viable organism and using a dead one. BCG will multiply in the host. One can recover BCG from an animal as long as a year or more after inoculation. This is not operative with an inanimate adjuvant. Thus with BCG one has not only multiplication but also persistence.

D. W. Weiss: Just one additional point of information: Some nonliving mycobacterial material, such as the methanol-extraction residue, can be recovered even 16 or 18 months after a single intraperitoneal injection of as little as 150 or 200 mg. Prolonged maintenance in the host is not restricted to living bacilli.

Zbar: You can culture tubercle bacillus from the spleen of the guinea pig very readily a month after an intradermal injection, and the cell walls that Dr. Ribi prepares are present in the animal for several months after.

Chase: Dr. Zbar and Dr. Rosenthal: What is the quantitative aspect of this? Actually the number of viable organisms plummets down in both mouse and guinea pig. That you can still get a few viable organisms out goes without saying. But what are the actual figures that you obtain when you isolate, related to the given dose that could have been isolated, say, 12 hours after injection?

Alexander: That is the key point: At about 10 days or so after BCG injection in the mouse, the level of circulating antigen is extremely low and quite undetectable; whereas with toxoplasmosis, the antigens can be found in the animal for their entire life.

Therefore, this arming-to-activation stage by contact with the antigen of the immune macrophage can occur in the animal with toxoplasmosis and cannot occur in the BCG-treated animal.

I didn't mention the experiments that can be done by just taking the serum from such animals.

Chase: If BCG or any other dead mycobacterium is suspended in paraffin oil, it can be found in, let's say the para-tracheal nodes and in other favorable sites for 2 or 3 years afterwards. So that shucking off is no less with the oil-suspended cells than it is with the initially living BCG.

Zbar: In terms of your questions, in the guinea pig and mouse, there are about 1 or 2 logs fewer bacteria after a month has elapsed than when the bacteria was inoculated. So if you inoculate 6 million bacteria intradermally, a month later there are about 600,000.

Unidentified speaker: How about 2 months?

Zbar: I don't know.

J. W. Kreider: Dr. Hanna and Dr. Zbar, I was intrigued by the germinal cell hyperplasia in the regional lymph node. Have you attempted to find out what's going on in the serum of these animals? Is there a rising antibody titer against the BCG and also against the tumor cell? What relationship, if any, does B-cell hyperplasia have with the so-called enhancement some of us have seen?

Hanna: I have also been extremely intrigued by this finding of a marked lymphoproliferative response with germinal center activity in the regional lymph nodes of animals from which tumors have been excised. It is more remarkable when one remembers that the metastases growing successfully in this lymph node will eventually kill the animal despite this presumably specific lymphoproliferative response. Maybe the recognition of tumor antigens, as brought to the node by metastatic cells, is different and results in a different response than that elicited by antigen which would be entering the node from the tumor site and draining in as processed antigen. We did not see this degree of lymphoproliferative response in regional nodes when we had metastases as well as processed antigen coming from the tumor site. The lymphoproliferative was seen only in the surgically excised animals in which the processed antigens of the tumor site were absent but the metastatic growth in the lymph node was still present. The overall response, however, in the excised animals may have something to do with the specific tumor antibody production. We have sent some of the serum to Dr. Borsos. Perhaps Dr. Borsos could discuss some of the preliminary data.

T. Borsos: We have tried 2 techniques: fluorescent antibody and C1 fixation and transfer. We could demonstrate antibodies by both techniques in these guinea pigs. What is bothersome at the present time is that we found antibodies both in those guinea pigs that are going to be cured by BCG treatment and those that are going to die after tumor excision.

Baldwin: At one point we looked at tumor-specific antibodies in animals immunized with irradiated hepatoma cells; with or without BCG, antibody response did not markedly increase.

Rosenthal: To answer Dr. Chase, when an antigen is enrobed in oil, this oil can be found in the septal cell of the lung for years afterwards and may cause granulomas to form, or even tumors. This is one of the objections to using oil in any vaccine.

Chase: I do not advocate oil, Dr. Rosenthal.

Sokal: This point is of considerable practical important to clinical immunotherapy. BCG is not an entirely innocuous organism. Patients can get into trouble with BCG; an acid-fast infection may develop in our patients whom we have exposed to BCG. Often I have put patients on streptomycin and isoniazid because I was afraid that a particular febrile illness they had might in fact be acid-fast disease. I want to know what is the danger period of developing an infection; when do I have to stop worrying about this, etc.?

Borsos: Dr. Waksman, one of the purposes of inviting you was not only to share your tremendous knowledge with us and our affection for you, but also perhaps to stimulate you, in a delayed way perhaps, or immediately,

to start thinking about and looking at the tumor immunotherapy problem, not just from the standpoint of T-cells, B-cells, and all the beautiful basic studies, but also from the standpoint of tumor immunotherapy which seems to have some special problems which appear, at least at the moment, unlike any other problems challenging us.

So if we were successful in stimulating you to start thinking of the experimental endpoint as immunotherapy, and to apply thereto your wonderful knowledge, I think we have won a big battle.

B. H. Waksman: I am pleased to report to you that this thought arrived finally in my head last week.

Weiss: Ladies and gentlemen, before we adjourn I would like to take the liberty of holding you for a few minutes. My purpose is to focus on 8 points which arose over and over again in today's discussion; by calling attention to them in closing, we might be able to pinpoint the most crucial aspects of this area of BCG and related compounds in tumor immunology which require immediate further investigation. I will not go into detail, but want merely to list these aspects.

1) We have not yet related in a satisfactory manner 3 crucial variables in dealing with a living vaccine like BCG: the starting amount of the material as it is administered; the viability of the organisms at the time of injection; and, most importantly, their intrinsic potential for growth in a particular host. When we speak of quantitation or dosage, these independently variable factors must be considered together.

2) When we speak of dosage, route, and strain of BCG, we also cannot discuss any one of these 3 parameters in a vacuum. Dose, route, and strain must clearly be interrelated. What is optimum dose for one route and one strain is not so for another route and another strain. There is an interaction here.

3) A very important point made today is that even some of the toxic or otherwise seemingly undesirable components of BCG, and perhaps of other nonspecifically active bacteria, might be needed to obtain a maximum antitumor effect. This is especially evident with regard to the elicitation of local delayed-hypersensitivity reactions which sometimes destroy neoplastic foci. What is desirable and undesirable in a bacterium must here be viewed in terms of the totality of host-tumor relationships with which one attempts to interfere therapeutically, not in terms of toxic or allergenic properties of one sort or another in themselves. A toxic agent may, as Dr. Ribi has pointed out, nonetheless play a vital role in a given interaction between host and tumor with regard to nonspecific immunologic interference.

4) Most likely the various crude mycobacterial materials that have been investigated consist of batteries of active principles with considerable overlapping and quite possibly also with a canceling out of the action of one by another. Major efforts must now be directed at attempts to isolate active molecular entities. This is almost a platitude!

5) Related to this is the next comment: There is a crying need for the establishment somewhere of a comparative testing program, under similar conditions and in a number of identical models, of the various agents which have been described as effective in stimulating immunologic capacity and resistance to tumors nonspecifically.

6) It must be considered that there is a kind of specificity even to "nonspecific" inflammatory responses; not just any inflammatory response or granuloma has therapeutic implications. On the other hand, it must not be forgotten that at least some nonspecific stimulators of resistance work without inducing, at least at the tumor site, any discernible granulomatous effect.

7) It should be emphasized that, whereas physical proximity between tumor cells and nonspecific stimulator may be requisite in some species and in some systems, it is not a necessity in other species and in other models.

8) Finally, there arises continuously the basic problem of predicting a priori whether a given specific and/or nonspecific immunologic interference will lead to enhancement or to resistance. Major emphasis must be given in the future to the monitoring of the immune response of a given host, in a given relationship with a given tumor, as a function of specific or nonspecific stimulation. It appears from preliminary observations on leukemia patients in our institution that such monitoring may yield clinically pertinent information. We may never be able to determine with certainty from animal models what parameters will lead to resistance rather than to enhancement against tumor cells in man, but we may be able to obtain this information from the individual patient during treatment. Clearly, we must take into account not only species but also individual variations from host to host, and any immunotherapeutic attempts in man in the future must take into consideration not only the desirability but also the necessity of obtaining, as one goes along, a clear picture of what is being done in terms of relative cellular and humoral immune activity.

With these several points, I hope that I have succeeded in at least delineating some of the major areas which came under discussion this afternoon. Certainly this does not make order of a very complicated field, but it may at least indicate where some of the major action lies at this moment.

HUMAN STUDIES

Discussion Leaders: Edmund Klein (Morning Session) and
Brigid Gray Leventhal (Afternoon Session)

Introduction: Immunotherapy of Cancer in Man, a Reality[1,2]

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SUMMARY—Induced allergic reactions (cell-mediated immunity) result in eradication, early diagnosis, and prevention of cancers and precancerous lesions of the skin. These approaches have been extended to other types of cancer (e.g., melanomas, lymphomas, breast cancers, and sarcomas) indicating general underlying principles. Newly or previously acquired immunity (immune memory) can be utilized for cancer therapy. Local immune reactions can be generalized to result in resolution of disseminated malignant tumors. Concurrent or sequential combinations of multiple, immunologically active agents (immunotherapy), X-ray therapy, cancer chemotherapeutic agents, and/or surgical procedures can be more effective than single treatment methods. Immunity transferred by white blood cell preparations from responsive to nonresponsive patients can be used for immunotherapeutic procedures.—Natl Cancer Inst Monogr 39: 139-161, 1973.

I HOPE that this and future conferences will contribute to "Tumor Immunotherapy in Man" becoming a reality. The purpose of this introduction is to provide background material, assessment of the current state of the art, and prospective views. I will not apologize for referring to material which I have presented before. Rather, I hope that a 10-year follow-up study on everything which I am presenting today will show the same results and validity. This material was presented 5 years ago and it is still valid. Hopefully it will hold up on a larger scale 10 years from now. By that time the phenomena and their statistical documentations should be definitively established.

A substantial amount of data which I will present may not comply with the standards of biostatistical evaluation. Nevertheless, malignant diseases by and large are irreversible unless exogenous factors are being brought to bear upon their course. While the clinical data presented this morning is anecdotal by formal statistical criteria, they are significant biologically, since their occurrences by chance are very unlikely, or cancer would not be as serious a problem as it is.

The observations on immunologic approaches to cancer are becoming sufficiently compelling to indicate the need for obtaining data by objective evaluation which are suitable for clinical appli-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

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cation in a predictable manner. It is with one objective in mind that we should approach the presentations this morning and this afternoon on the clinical aspects of immunologic manipulations in patients with cancer. Are we presently at the stage where we can decide whether the data obtained so far provide adequate justification for entering a Phase I and planning a Phase II level of studies on immunotherapy?

These introductory remarks are intended to put previous findings in cancer immunotherapy into perspective with the present intensive work on BCG and with the future directions of research in tumor immunotherapy. The findings on which these thoughts are based come from collaborative work of a group of my associates at Roswell Park Memorial Institute and other institutions.[3] We had the opportunity of looking at malignant lesions of or in the skin, which allowed us to study the interaction of host, tumor, and exogenous factors.

Skin cancers provide investigative flexibility which is not available for studies of cancer in the deep-seated tissues. We can manipulate cutaneous tumor systems so that we can introduce minimal interference with the internal milieu of the host. We thus can observe mechanisms obscured by more intensive therapeutic procedures used for deep-seated cancers. Not only are we able to look at malignant lesions in the skin like specimens in a test tube, but also we can do so in vivo, in the intact organism, marshalling whatever measures of defense it can mount!

I believe it was for these reasons that we noted immune as well as other defense mechanisms earlier in our work, when we studied the local effects of cytotoxic chemotherapeutic agents. We applied cytotoxic agents directly to cutaneous tumors to reach high, local concentrations, which of course result in relatively low levels in the blood and other tissues. This approach contrasts with parenteral administrations of anticancer drugs which require large doses in the hope that a small fraction will reach the tumor. In this way we were able to preserve the integrity of the host and at least some of the defenses which the host can muster.

It became apparent that the balance could be shifted in favor of the host and against the tumor. When this was accomplished, defense mechanisms of the host, theretofore ineffective against the tumor, became highly effective. One of these mechanisms was recognized as the immune system, particularly the cell-mediated component. Another line of defense appeared to be the monocytes with or without the synergistic actions of lymphocytes or other white blood or tissue cells. Although a competent immune system can induce an intense macrophage activity against tumors or other nonautologous materials, immune activation does not appear to be essential. Collaborative studies by Djerassi and his associates[4] and our group have clearly shown that introduction of concentrated human monocytes result in tumor regression. This was demonstrated in vivo and in vitro with the use of monocyte preparations obtained from the peripheral blood of humans or the peritoneal cavity of experimental animals. Analogous data were obtained with monocyte preparations obtained from normal individuals or those with malignant diseases.

The increased antitumor effects of combining several antigenic agents for immunotherapy, chemotherapy, or radiation therapy may be due to increased activation of macrophages by several agents or different therapeutic modalities. These considerations can be illustrated by a patient who represents an experiment of nature. The patient had a low-grade, chronic, tuberculous infection for several decades—an analogue of continuous BCG administration. The course and management of this patient indicate the need for a comprehensive approach to patient care in malignant diseases at the present stage of our knowledge. If individual therapeutic modalities are not adequate, we may have better results when we combine ≥ 2 forms of treatment. Thus immunotherapeutic approaches were combined concurrently or sequentially, with chemotherapy, surgery, and radiation therapy. The patient under discussion (fig. 1) had lupus vulgaris of > 20 years' duration. In

[3] Sloan-Kettering Memorial Hospital, New York, N.Y.; Children's Cancer Research Foundation and Harvard Medical School, Boston, Mass.; Mercy Catholic Medical Center, Philadelphia, Pa.; and the National Institutes of Health, Bethesda, Md.

[4] Mercy Catholic Medical Center.

one of the several areas of involvement by lupus vulgaris, she developed a squamous cell carcinoma which remained untreated for an unknown number of years. She had a large squamous cell carcinoma involving the right shoulder and extending onto the axilla, arm, chest, and lateral aspect of the tail of the breast. Exhaustive review failed to demonstrate metastatic and/or deeply invasive malignant disease. Evidently, despite its largeness, this tumor remained localized for a protracted time. The localization of this tumor and lack of metastases are most likely related to immunopotentiation associated with the long history of lupus vulgaris—an analogue of BCG vaccination on a continuous and intense basis.

When the patient presented at our Clinic, the tumor was inoperable and considered unsuitable for radiation therapy. At the same time, immunotherapeutic approaches were initiated; administration of purified protein derivative (PPD) (fig. 2) resulted in a strongly positive response in the normal skin at the challenged site. In addition, the sites of lupus vulgaris involvement showed intense flare reactions, including the area involved by malignant diseases. Chemotherapy with bleomycin was also instituted but had to be administered carefully in view of the hazards of pulmonary fibrosis, particularly in the presence of tubercular disease.

Combined therapy including intralesional PPD and bleomycin markedly regressed the tumor (fig. 3), which was replaced by normal skin. This represents a process of selectivity in which the malignant epidermal cells are undergoing destruction, while the normal cells are growing and behaving normally in other respects. However, there was still a considerable amount of residual tumor. Systemic bleomycin had to be discontinued because of potential side effects. In its place, 20% 5-fluorouracil (5-FU) was administered topically for approximately 3 months, while immunostimulation was maintained by PPD administration.

The tumor underwent resolution (fig. 4), and was subsequently explored by multiple biopsies; the area was found to be free of tumor. After débridement, parts of the denuded area were grafted (fig. 5). The site became fully resurfaced, and the patient has remained free of disease for approximately 2 years (fig. 6). This is an example of combining several therapeutic modalities, although it is difficult to determine whether immunotherapy or chemotherapy, or their combined effects, were the critical factors in bringing about the favorable outcome of this problem which was untreatable by standard therapeutic measures.

It has been previously shown (*1-6*) that induction of delayed-hypersensitivity reactions at sites of premalignant and malignant epidermal lesions selectively eradicated neoplasms. Early lesions, otherwise clinically undetectable, become apparent by their response to immunologic challenge, thus permitting diagnosis and, at the same time, eradication. Also, immunologically induced destruction of premalignant keratoses prevented their progression to squamous cell carcinomas. Thus the incidence of new, clinically significant, malignant lesions was reduced in patients who otherwise continue to develop increasing numbers of tumors.

Subsequent studies (7) indicated that immunologic principles analogous to those developed in epidermal neoplasms pertain to broader aspects of tumor biology. Immunotherapeutic approaches to a spectrum of malignant diseases in man (including lymphomas, sarcomas, melanomas, adenocarcinomas, mycosis fungoides, and a number of other types of cancer) were initiated. Our findings and those of others (*7, 8*) demonstrated that cell-mediated challenge reactions at a single tumor site may initiate regression in distant, unchallenged lesions in the skin and at other sites.

Another important immunotherapeutic approach utilized immunologic memory in patients whose impaired immunocompetence did not permit the development of cell-mediated immunity to new antigens. We observed that the concurrent administration of ≥ 2 sensitizing agents synergistically potentiated the antitumor activity of cell-mediated immune reactions. Furthermore, we found that similar synergistic antitumor effects could be obtained by combining chemotherapy and/or radiation therapy with immunotherapeutic approaches.

Selective tumor eradication by immune mechanisms was first demonstrated in patients with primary epidermal neoplasms (*1–3*). These neo-

plasms included multiple, extensive, premalignant epidermal lesions (keratoses and leukoplakia), intractable basal cell carcinomas, and squamous cell carcinomas. The neoplastic lesions underwent successive stages of delayed-hypersensitivity reactions. During or after involution of tumors, granulation tissue formed and re-epithelialization proceeded. Thus the site of tumor involvement was being resurfaced at the same time as the tumor was involuting.

Epidermal tumors were studied during the past 10 years in a group of > 100 patients with multiple skin cancers and premalignant lesions. Follow-up studies on an initial group of 24 patients for periods of ≥ 5 years showed a $> 95\%$ rate of eradication of neoplasms in $>5{,}000$ lesions. The initial study group of patients had intractable diseases for which standard modalities were inadequate (e.g., multiple basal cell carcinoma nevoid syndrome, late radiation dermatitis with malignant degeneration, xeroderma pigmentosum, arsenical dermatitis, and severe skin damage with multiple malignant lesions due to excessive exposure to artificial ultraviolet radiation or from sunlight). Fifty-four patients with less severe diseases were followed during the past 3–5 years; results were essentially similar, although definitive evaluation will require continued follow-up observation. The 37 patients in the third group have been under variable observation periods ranging from 6 months to 2 years. Meaningful follow-up will require at least an additional 3–5 years. The data obtained so far, however, appear to agree with those obtained in previous studies.

The information obtained from these studies demonstrates that induced delayed-hypersensitivity reactions provide a basis for lasting tumor eradication. Selective responses to immune challenge occur in early lesions, which are otherwise undetectable. As a result of the inflammation reaction during challenge response, these early tumors become apparent and undergo resolution. Clearly these observations are limited by tumor size; it is likely that early or small tumors, which fail to show a grossly observable reaction, are also responding to immune challenge and undergo resolution. Therefore the incidence of subsequent, clinically more significant neoplasms is diminished. Reduced incidence of malignant and premalignant lesions is clinically significant, since skin tumors are frequently multiple. These considerations pertain to the common, sun-induced skin tumors as well as to the much rarer congenital disorders.

Patients with multiple skin cancer syndromes usually develop increasing numbers of tumors during the natural course of the disease. Standard therapeutic procedures (e.g., surgery and radiation) are naturally limited to apparent lesions and cannot be applied to reduce the subsequent incidence of tumors. Since patients with multiples cancers are closely observed, data on the incidence of lesions are available for at least 5 years before and after immunotherapy. The incidence of tumors in 7 patients with xeroderma pigmentosum requiring as many as 100 surgical procedures per year was reduced by immunotherapy so that surgery was rarely, if at all, needed during observation periods ranging from 3 to 7 years. Most lesions arising after an initial course of immunotherapy could be managed by subsequent immunologic challenge reactions and did not require surgery, since they were not allowed to progress to advanced stages.

These findings show that appropriate immune reactions can eradicate undetectable tumors, both at premalignant and malignant stages, and prevent the incidence of neoplasms. These preventive and therapeutic processes can occur without grossly apparent manifestations of an immune reaction.

To induce a cell-mediated immune challenge reaction resulting in therapeutic effects on tumors, the patient has to have adequate immunocompetence for a particular antigen. A number of patients with multiple epidermal tumors were unable to develop sensitivity to the several agents under study. Most of these patients, however, had retained immunologic memory for antigens which they had developed before impairment of immunocompetence. Sensitivity to dinitrochlorobenzene (DNCB) was induced in 57 of 68 patients with cancers involving the skin. Of 11 patients who failed to develop hypersensitivity to DNCB, 9 showed immunologic memory to ≥ 1 microbial antigens, although the intensity of the challenge reactions varied considerably.

Cell-mediated challenge based on immunobiologic memory was found (*9*) to produce selec-

tive antitumor effects and to result in eradication of premalignant and malignant epidermal tumors, cutaneous lesions of lymphomas, and other nonepidermal cancers. Our studies indicated considerable variation in the rate and degree of antitumor responses to cell-mediated challenge with microbial antigens. Lesions in mycosis fungoides regressed after a single injection of PPD, although usually multiple injections were required. These findings suggest a general relationship of cell-mediated immunity to tumor biology.

From a more practical standpoint, these findings permitted the extension of immunotherapeutic approaches to patients with extensive malignant disease and impaired immunocompetence who could not develop cell-mediated immunity to new agents. The data further indicate that sensitizing agents of relatively large molecular weight can produce reactions similar to those induced with compounds of small molecular weight. These observations also raise the possibility that exposure to microbial or other antigens may increase the effectiveness of the immunologic surveillance mechanism against tumors as well as infectious diseases.

Antitumor effects of cell-mediated immune challenge reactions have been extended to a broad range of malignant tumors, including squamous cell carcinoma, basal cell carcinoma, reticulum cell sarcoma, mycosis fungoides, Kaposi's hemorrhagic sarcoma, lymphangiosarcoma, malignant melanoma, and adenocarcinoma of the breast as well as certain premalignant lesions, such as leukoplakia, keratoses, and lentigo. Responses in neoplastic lesions involving the skin and subcutaneous tissues included complete regressions, partial regressions, temporary arrest of progression, and lack of observable response. Regressions of lesions were induced by challenge reactions following haptene-induced immunity or on the basis or immunologic memory to microbial antigens, or both.

Cell-mediated immune challenge resulted in selective reactions and regressions of $> 50\%$ of lesions of mycosis fungoides (18 patients) and reticulum cell sarcoma (7 patients). The data showed that immune challenge effects on lesions of mycosis fungoides or reticulum cell sarcoma were similar to those on epidermal tumors and occurred at concentrations of the sensitizing agent at which minimal or no reactions were produced in the normal tissues.

Selective antitumor effects of local challenge reactions could also be demonstrated in patients with other types of disseminated, solid, malignant diseases involving the skin and subcutaneous tissues. Of 30 patients with at least some cell-mediated immunocompetence, 16 showed more intense challenge responses at tumor sites than in normal tissues. Effects varied considerably from lack of response to complete involution in different patients and at times in the same patient. The variations in tumor response indicate the need for determining the mechanisms which account for these differences in the antitumor effects of cell-mediated immune challenge reactions.

Challenge of a single lesion in some patients was associated with reactions at distant, unchallenged lesions. In 26 of 108 patients, lesions at various distances from the challenged tumor site underwent partial to complete involution after they had shown the equivalent of delayed-hypersensitivity reactions. These observations suggest that a systemic factor produces reactions at sites distant from the challenged area and can be compared to the "flare" reaction at distant sites of tuberculosis involvement when patients with cutaneous tuberculosis (lupus vulgaris) are challenged with PPD.

Immunopotentiation was attempted in patients with markedly impaired immunocompetence who failed to respond to the standard methods of eliciting a cell-mediated immune response. Patients who had failed to respond to standard doses of PPD, *Candida* extract, or other microbial agents reacted to higher concentrations of the antigens. Once a response to one antigen was elicited, responses to other antigens also became apparent. Consequently, the higher levels of antigens could then be used for therapeutic purposes (figs. 7–11).

Concurrent challenge with ≥ 2 sensitizing agents increased the intensity of challenge reactions as compared to the effects of single antigenic agents. A challenge response could be demonstrated when 2 sensitizing agents were used concurrently at the same site at concentrations at which either antigen alone did not

produce an adequate reaction (fig. 12). These observations were explored in neoplasms which had failed to respond to challenge with a single sensitizing agent. The concurrent use of multiple sensitizers results in tumor eradication when a single sensitizing agent is ineffective (figs. 13–15).

The observation periods after immunopotentiation by increasing the concentration of antigens or combining ≥ 2 agents are too short and the numbers of patients studied too small to allow more than preliminary conclusions. They do, however, suggest a common trend. Challenge reactions based on immunologic memory resulted in regression of treated and untreated lesions; responsiveness appeared related to the intensity of the cell-mediated challenge response; and the intensity of that response could be increased by increasing the concentration of antigen or concurrent administration of ≥ 2 antigens. These observations may be of significance in relation to BCG, *Citrovorum parvum*, or other microbial preparations. The preparations of whole microorganisms clearly contain numerous antigens which are administered concurrently. Furthermore, living organisms as a result of growth produce increased amounts of antigens. Thus the above observations provide additional rationale for exploring living microbial preparations for immunotherapeutic approaches in malignant diseases.

As indicated by experience demonstrated by the patient (figs. 1–6) discussed above, combinations of chemotherapy and/or radiation therapy with immunotherapy justify further attention. Local administration of antimitotic agents (e.g., 5-FU) did not interfere with the challenge response to DNCB, triethylene-imino-benzoquinone (TEIB), or microbial antigens (7). Concentrations of topically administered 5-FU 1000-fold greater than the level attainable by systemic administration enhanced rather than inhibited the challenge response to DNCB or TEIB. Note that systemic administration of 5-FU at standard doses inhibited specific cell-mediated immune reactions. Therefore, it would appear either that locally administered antimitotic agents do not adversely affect mature mononuclear elements (lymphocytes and macrophages) or that damaged cells are replenished from central lymphoid organs. At concentrations at which 5-FU alone or DNCB alone do not produce reactions in tumors or normal skin (control), a therapeutic response can be demonstrated when both agents are administered concurrently to the same site (figs. 16, 17). Protracted use of 5-FU at the concentrations used ($\leq 1\%$) did not produce reactions, while DNCB at low concentrations induced a reaction of low intensity which took up to 5 days to develop. Apparently the administration of 5-FU enhanced the effects of the sensitizing agent.

Concurrent use of antimitotic agents and challenge responses was studied in 23 patients; it resulted in eradication of tumors which had failed to undergo resolution when the antimitotic agent or the sensitizing agent was used alone at comparable concentrations over protracted periods. These observations suggested that concurrent antimitotic chemotherapy and immunologic challenge may be developed as a clinically valuable approach to cancer therapy.

Treatment of malignant disease frequently requires concurrent, sequential use of various therapeutic modalities to assure optimal clinical management. We subsequently found that therapeutic results could be obtained by combining local or systemic chemotherapy and/or low doses (100 rads) of radiation therapy with immunotherapy (figs. 18, 19), which could not be obtained with single modalities: The findings obtained so far indicate the need for further exploration.

Extensive malignant diseases involving the skin were treated with a combined approach including chemotherapy, radiation therapy, immunotherapy, and surgery. Combination therapy included immunotherapy utilizing challenge reactions at tumor sites, based on either previously established delayed hypersensitivity (e.g., microbial antigens) or de novo sensitization to small organic molecules (e.g., DNCB). Clinical management of 23 patients with extensive cutaneous neoplasia, followed for up to 8 years, involved surgical removal of tumors requiring immediate attention, followed by immunotherapy, topical chemotherapy, or both. These modalities permit the removal of advanced superficial as well as early, otherwise undetectable, malignant disease. This facilitated subsequent

surgery and reduced the incidence of recurrences. Similarly, preparation of donor sites by chemotherapy or immunotherapy before grafting reduces the possibility of transferring malignant lesions.

Effective combination of systemic chemotherapy with local or systemic immunotherapy depends on the state of immunocompetence and therefore requires either selection of chemotherapeutic agents which do not suppress immunocompetence or a regimen which permits retention of immunocompetence. Chemotherapeutic agents having minimal effects on immunocompetence include bleomycin, azaribine, triacetylazauridine (TAU), and vincristine among others. Systemic bleomycin, used to treat extensive inoperable squamous cell carcinomas, caused minimal, if any, impairment of the patient's immunocompetence. Tumors which did not adequately respond to bleomycin alone or to immunologic challenge reactions alone underwent resolution when systemic bleomycin was used in conjunction with local cell-mediated immune challenge. The further addition of local chemotherapy resulted in compete regression of tumors, which permitted skin grafts and complete healing.

Lesions of Kaposi's hemorrhagic sarcoma which had failed to respond adequately to systemic or local administration of vinblastine or to immunologic challenge reaction responded when vinblastine was used systemically in conjunction with local immune challenge reactions.

TAU was used systemically in patients with mycosis fungoides at the eczematoid and plaque stages. When local cell-mediated immune challenge reactions were elicited at sites of lesions while TAU was being administered systemically, rapid resolution of the challenged lesions occurred, while unchallenged (control) lesions persisted. These studies further indicate the feasibility of use of concurrent systemic chemotherapy and immunotherapy. Patients with mycosis fungoides who had failed to respond adequately to chemotherapy and/or immunotherapy showed resolution of lesions when low, usually ineffective, doses of ionizing radiation (100–250 rads) were added to the therapeutic regimen (*10*).

The gross appearances of observable involuting malignant lesions resemble each other, whether immunotherapy, chemotherapy, or radiation therapy is used singly or combined. The microscopic appearances also resemble each other in that they invariably contain monocytes as a significant component of the cellular infiltrate at some stages of tumor resolution. Our previous studies (*7, 11–13*) and those of others (*14*), furthermore, suggested that monocytes have primitive recognition mechanisms which permit selective destruction of "foreign" materials including autochthonous tumors. Studies were done in collaboration with Djerassi to determine the effects of local administration of concentrated monocyte preparations obtained from peripheral blood of normal human donors and patients with cancer.

Local administration of monocyte preparations, both with and more notably without immunologic (lymphokinin) activation, resulted in at least temporary regressions of cutaneous or subcutaneous metastatic lesions of mammary adenocarcinoma, infiltrates of mycosis fungoides, and epidermal tumors (figs. 20, 21). These observations are consistent with the data obtained by Zbar et al. (*15*) who showed that the effects of macrophage preparations closely resemble those of BCG administration in the hepatoma-bearing guinea pig system. It is too early to evaluate the therapeutic implications of monocytes in human cancer. It appears, however, from observation in vitro, in cell culture systems, in experimental animals, and now in man, that studies on the antitumor activities of monocytes provide important information on the mechanism of what may be a final common path in selective tumor cell destruction regardless of the initial steps which precede it. The initiating mechanism for activating macrophages may require one or more of several factors, some of which may not depend on the immune system.

The data presented here and the presentations later today indicate that immunotherapeutic approaches to malignant disease in man are warranted. These observations are consistent with the findings of Zbar et al. in the tumor-bearing guinea pig (*16*), the first animal model for the systemic study of immunotherapeutic approaches to cancer. Their findings have been widely confirmed and extended.

The findings described here should be viewed as exploratory and suggest feasibility and avenues justifying further pursuit, rather than as therapeutic procedures. Since immunotherapeutic approaches apparently pertain to a spectrum of neoplastic diseases in man, it may be worthwhile to explore effects of cell-mediated challenge reactions in a variety of tumors which have not yet been investigated in this respect.

The findings in metastatic and disseminated lesions indicate that at least palliative effects may be obtained at stages at which other methods of treatment have been exhausted. Furthermore, significant toxicity or generalized anaphylactic reactions have not been found in studies on $>$ 150 patients for periods of up to 10 years. On this basis, further studies on immunotherapeutic approaches to disseminated malignant diseases would appear to be medically and scientifically warranted.

Immunologic memory as a basis for inducing antitumor effects by immunologic challenge reactions may be of theoretical as well as practical significance. From a practical viewpoint, it permits an immunotherapeutic approach to patients whose immunocompetence has been impaired in respect to their ability to recognize new antigens. We have found that this impairment frequently occurs before memory for immunologic events prior to the onset of malignant disease is lost. Since most antimicrobial test antigens are relatively nontoxic, the parenteral administration of these agents is under exploration and appears to provide results analagous to those obtained on local or cutaneous administration.

The observations that ≥ 2 sensitizing agents used concurrently produce more intense antitumor effects than single agents also have theoretical as well as practical implications for immunotherapy approaches. Our observations indicate that the combined administration of ≥ 2 antigens produces delayed-hypersensitivity responses in normal tissues as well as at tumor sites at concentrations markedly below those at which single antigens elicit challenge reactions. A mutual adjuvant response between 2 unrelated antigens evidently enhances the manifestations of the reactions to these agents where none can be demonstrated otherwise. Possibly, similar considerations pertain in regard to eliciting a response to an otherwise weak or inadequate tumor antigen. The presence of several antigens may, therefore, initiate a response which includes a reaction against the tumor antigen. This may also be so when a single antigen induces a differentially intense reaction at a tumor site under conditions at which no reaction is evident in normal tissues. Since the normal tissue contains only the administered sensitizing agent, at a minimal concentration, it may resemble the situation in which single antigens fail to produce responses by themselves, but can elicit reactions when ≥ 2 are present simultaneously. Similarly, the tumor antigen alone fails to elicit an immune response but, in combination with another sensitizing agent, may provide adequate conditions for an immune reaction against both the sensitizing agent and the tumor antigen.

Concurrent administration of ≥ 2 sensitizing agents to tumor sites produces selectivity intense reactions which result in tumor destruction, as compared to the minimal reversible changes observed in normal tissues under the same conditions. The retention of selectivity in the presence of multiple antigens for a tumor antigen is analogous to increase in the reactivity when additional antigens are added, either in the normal tissues or at tumor sites. These observations may be significant in respect to the effects of complex antigenic systems of intact microorganisms, such as BCG, which contain a multiplicity of antigenic components.

It is of further interest that the dose-response relationship indicates that the intensity of cell-mediated immune challenge reactions is increased as the concentrations of the sensitizing agents are increased, once the threshold for inducing a detectable reaction has been reached or exceeded.

While the studies presented indicated the feasibility of immunotherapeutic approaches to several types of malignant diseases, further pursuit of failures of this approach will provide meaningful information on obstacles to the more effective and wider application of immunotherapy. Intensive study is also indicated on the relatively small proportions of patients in whom, so far, immunotherapeutic approaches have been

found to exert clinically significant antitumor effects. Further studies in this area may be of significance in tumor immunology, as well as provide information relevant to infectious diseases and other normal and pathologic processes in which immune mechanisms play a significant role. While the tumor system provides a challenging area for investigating complexities of delicately balanced immunologic systems, the converse is of major importance, since progress in the immunologic aspects of cell biology and the role of noncellular mediators and their regulatory mechanisms are of fundamental as well as applied importance.

Preliminary studies have been initiated to determine the effects of combining immunotherapeutic challenge regimens with X-ray therapy. Considerations similar to those pertaining to combining chemotherapy with immunotherapy pertain to these studies on combining radiation therapy with immunotherapy. Approaches to combining several agents with different modes of action against malignant tumors may therefore pertain to the combination of various treatment modalities in order to obtain optimal effects in the clinical management of malignant disease.

Selective destruction of tumor cells due to delayed-hypersensitivity challenge reactions at tumor sites is a complex phenomenon. It may involve both cellular and humoral factors. Since tumor regression is observed in the absence of discernible effects on the surrounding normal tissues, these factors must be selective or specific. Besides immunologically specific factors, other, not necessarily specific factors, including noncellular mediators and cellular components of the immune reaction, may be involved. In addition to recognized immune phenomena, therefore, other discriminatory mechanisms may be significant.

A delayed-hypersensitivity reaction involves local accumulation of mononuclear cells in addition to extravasated plasma proteins. Both cellular and noncellular components of the delayed-hypersensitivity reaction can be expected to take part in the observed destruction of tumor cells when the reaction is induced at the site of a tumor. The following factors seem to play a role: 1) The macrophages are the ultimate effectors of tumor cell destruction. 2) The selectivity for tumor cell destruction is mediated by *a*) a recognition mechanism peculiar for the macrophage, *b*) antibodies cytophilic for the macrophages, or *c*) antibodies interacting with tumor-specific antigens on tumor cells, thus earmarking the tumor cells for interaction with the macrophages. Preliminary experimental observations by us and by others support this view (*7, 11–14*). Peritoneal macrophages from rats obtained after BCG sensitization and PPD stimulation will selectivity destroy a number of different syngeneic rat tumors in vitro (*11, 13*). Serum components from tumor-bearing animals have been found to influence profoundly this in vitro interaction between macrophages and tumor cells (*12*). Not only can such sera accelerate the destruction of tumor cells by macrophages from stimulated animals, but also macrophages from nonstimulated animals will destroy tumor cells in the presence of serum from tumor-bearing animals. Thus we have evidence that macrophages can by themselves act as effector cells in tumor cell destruction and furthermore that serum factors can markedly effect the action of macrophages on tumor cells and macrophages.

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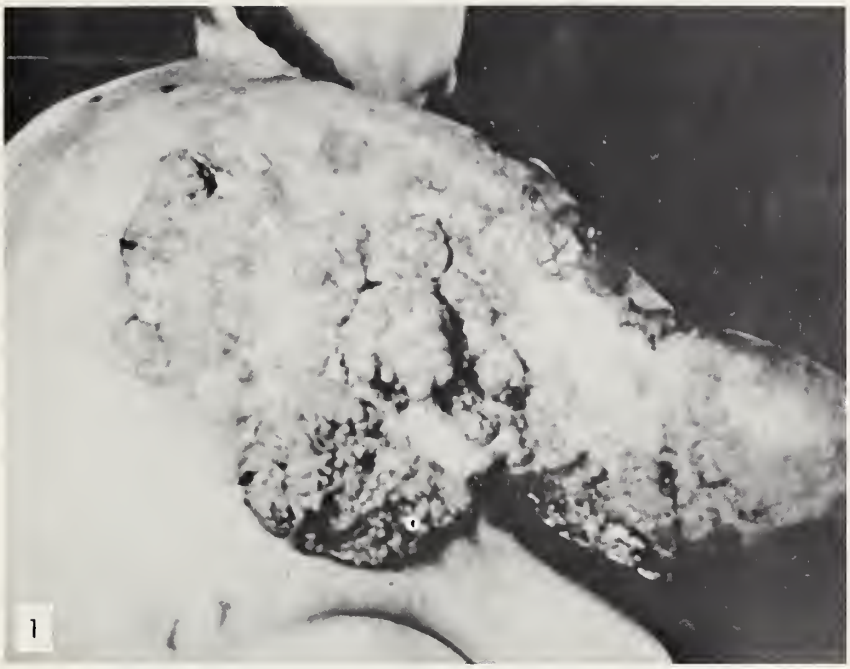


FIGURE 1.—Patient with extensive cancer (squamous cell carcinoma) before therapy. In view of its largeness, this cancer could not be treated by established methods such as surgery or X-ray therapy. A combination of chemotherapy and immunotherapy was used. The area was then grafted by plastic surgical procedures.

FIGURE 2.—Same area as shown in figure 1, after immunotherapy with PPD which was injected into and around the lesion while patient was on systemic bleomycin. Considerable degree of recovery has occurred but substantial amounts of tumor remain. Topical 5-FU (20%) was added.

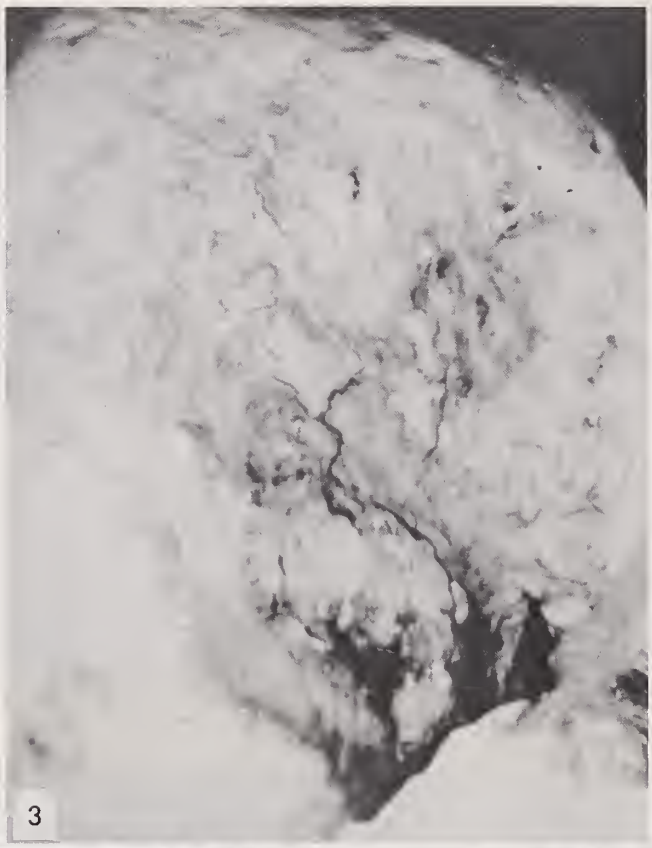


FIGURE 3.—Addition of topical 5-FU results in selective necrosis of residual tumor.

FIGURE 4.—Same area as shown in figures 1–3, after topical administration of 5-FU combined with intralesional administration of PPD. Area previously involved by tumor looks necrotic at this stage but is free of cancer. Previous attempts with bleomycin, 5-FU, or immunotherapy used alone did not give adequate results.

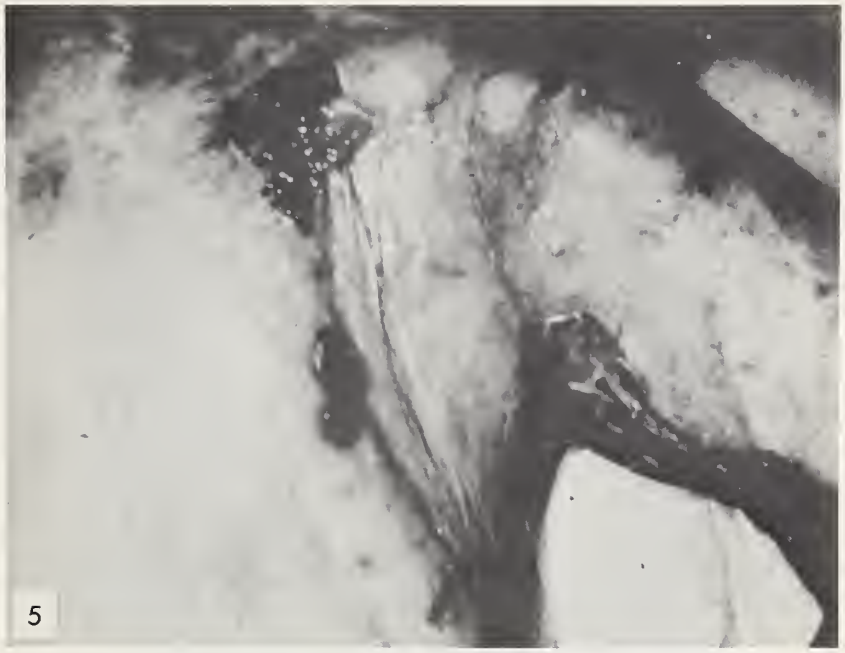


FIGURE 5.—After sites had been cleared of cancer, skin grafts were applied to cover denuded area.

FIGURE 6.—Complete resurfacing of sites previously involved by tumor. Patient remains free of disease >2 years.

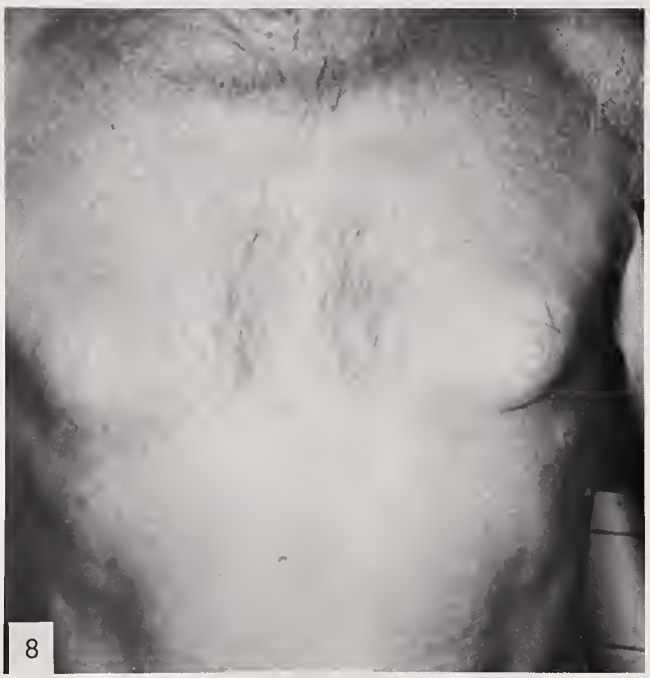
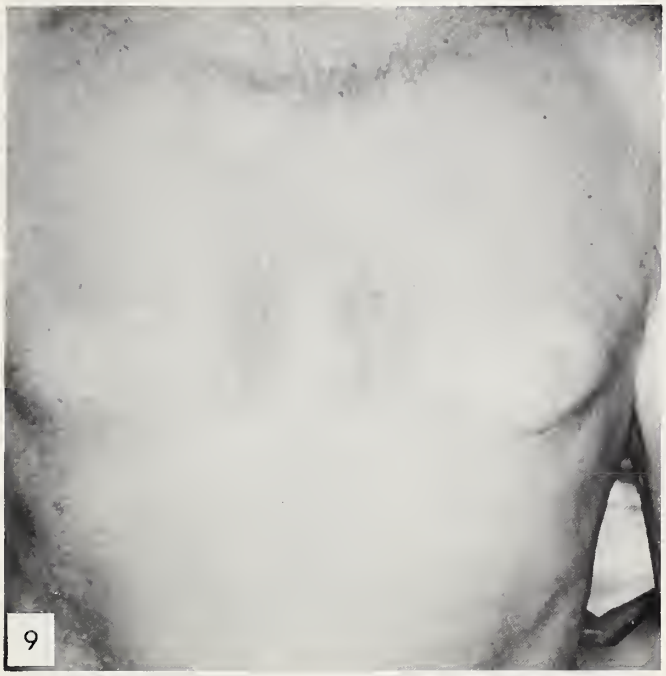


FIGURE 7.—Patient with mycosis fungoides at erythrodermic exfoliative stage. Before treatment, patient was not responding to battery of delayed-hypersensitivity skin tests, including second-strength PPD. Minimal response was induced by intradermal administration of 20,000 U of PPD.

FIGURE 8.—Same patient as shown in figure 7, before immunotherapy.



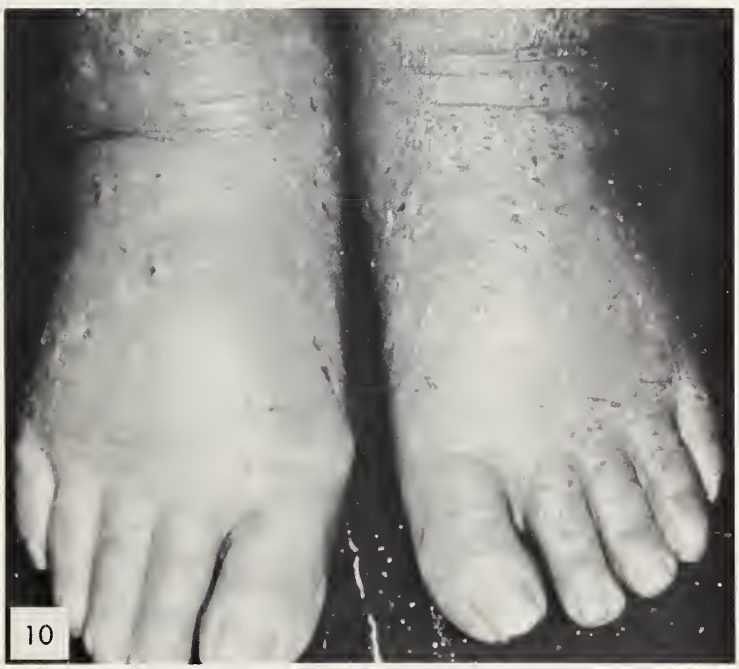
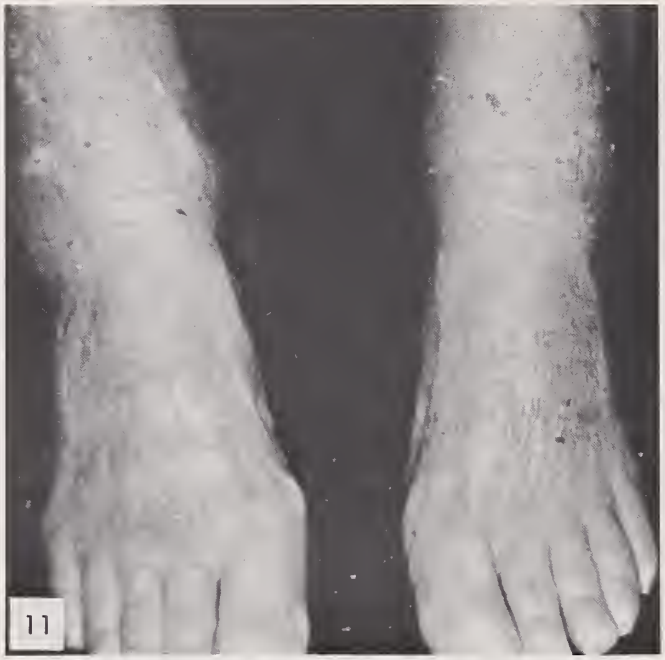


FIGURE 9.—Same patient as shown in figure 8, after topical administration of ointment containing 100,000 U of PPD/g. Approximately 3–5 g was applied twice daily to entire body for 6 weeks. Patient's skin has been cleared up completely. Multiple biopsies have failed to show residual mycosis fungoides. Patient has been free of disease for >1 year.

FIGURE 10.—Same patient as shown in figure 9, before treatment. Feet and ankles show extensive swelling and exfoliative erythrodermia.



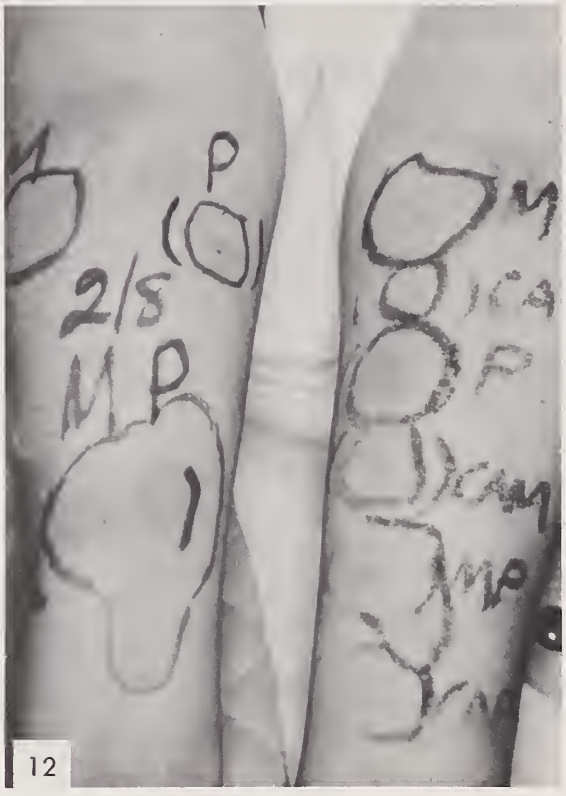


FIGURE 11.—Same patient as shown in figure 10, after treatment (described in fig. 9).

FIGURE 12.—Patient showing challenge reaction to a number of microbial antigens. Site at which PPD and mumps vaccine were administered concurrently shows far more intensive reaction than sites to which individual antigens were administered as single preparations.

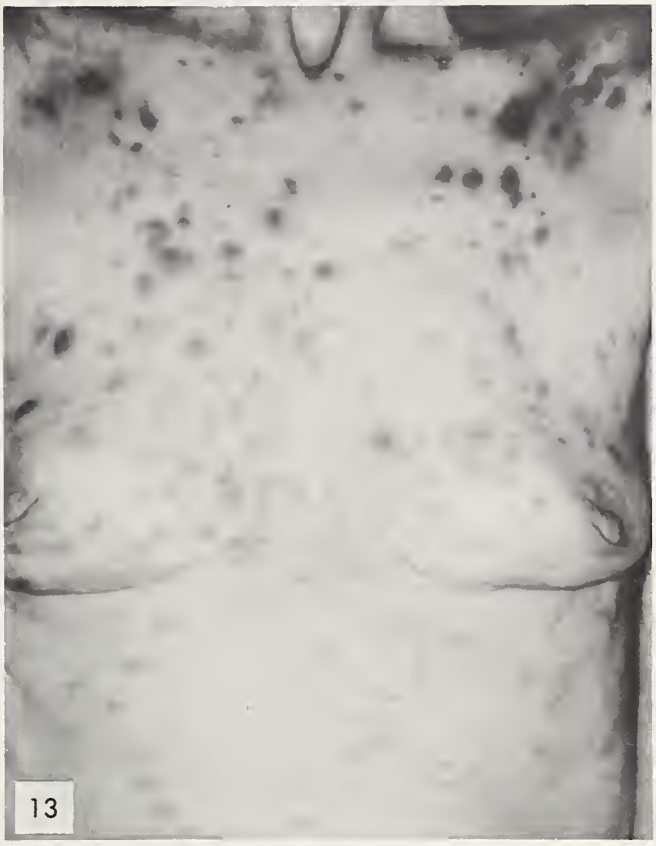
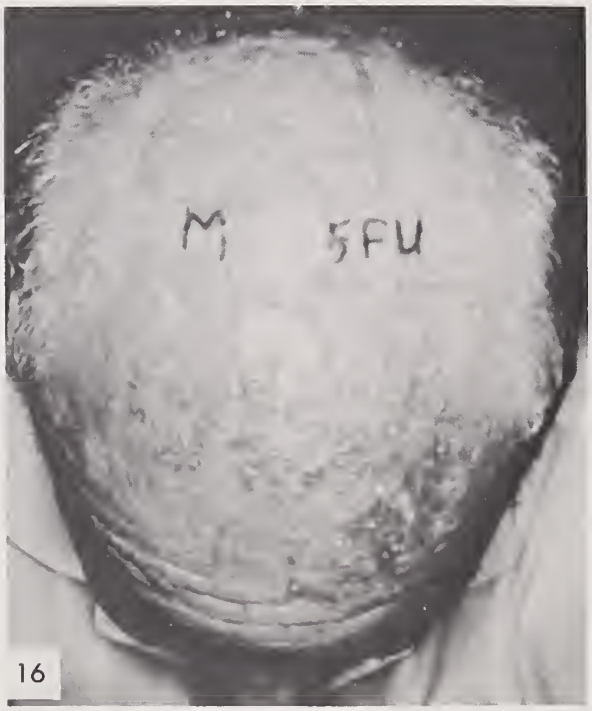


FIGURE 13.—Patient with reticulum cell sarcoma before immunologic challenge.

FIGURE 14.—Same patient as shown in figure 13, with extensive mycosis fungoides after reaction at sites distant from challenge, resulting in resolution of challenged and unchallenged lesions.

FIGURE 15.—Same patient as shown in figure 13, with reticulum cell sarcoma after challenge to single area and resolution of lesions of challenged and distant unchallenged sites. Square area to which challenge was applied revealed residual post-reactive pigmentation. Sites of previous involvement of challenged lesions also retain residual pigmentation free of tumor cells.



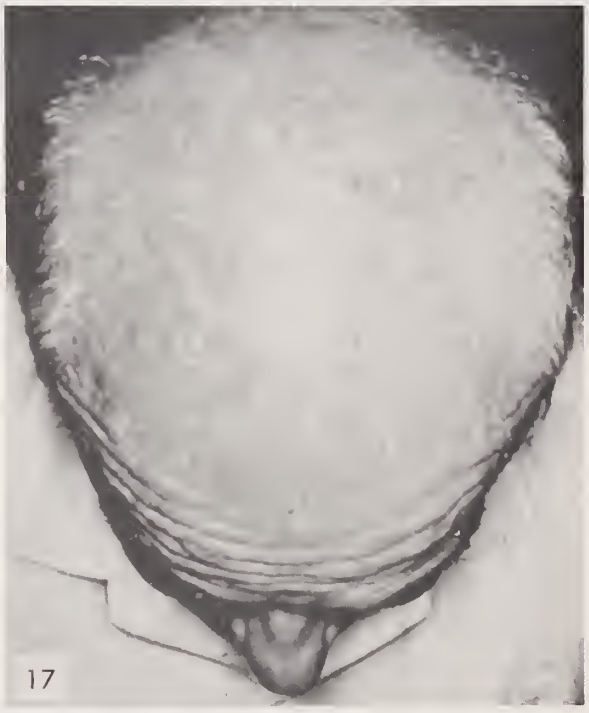


FIGURE 16.—Patient with actinic keratoses and multiple squamous cell carcinomas which had failed to respond to topical chemotherapy or immunologic challenge with DNCB.

FIGURE 17.—Same patient as shown in figure 16, showing complete resolution of lesions at sites where DNCB (immunotherapy) and topical 5-FU (chemotherapy) were combined concurrently, while control sites (i.e., DNCB or 5-FU above) continue to show lesions.

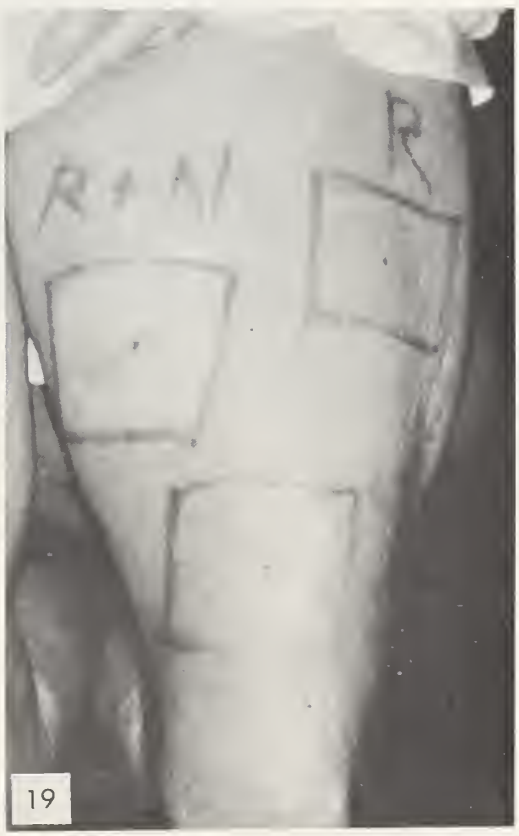


FIGURE 18.—Patient with mycosis fungoides at the erythrodermic exfoliative stage after remission lasting 3 months induced by electron-beam therapy (3000 rads). Marginal response to chemotherapy with TAU (6 g/day) for 3 months. Areas 1, 2, and 3 before treatment (described in fig. 19).

FIGURE 19.—Same patient as shown in figure 18; 2 weeks later showing clearing after combination of immunotherapy and superficial X-ray therapy (100 rads). *Sublegends:* Area #1—immunotherapy above showing minimal effects; Area #2—immunotherapy combined with radiation therapy (single dose of 100 rads) resulting in complete clearing; and Area #3—radiation therapy (single dose of 100 rads) showing no change.

FIGURE 20.—Nodules of recurrent carcinoma of breast on anterior chest wall after radical mastectomy, adrenalectomy, and chemotherapy. Inferiorly located lesion was infiltrated with approximately 50,000,000 monocytes obtained from peripheral blood of normal human donor.

FIGURE 21.—Same area as shown in figure 20, within 24 hours of injection of concentrates of normal human monocytes. Nodule of breast cancer has decreased in size; exophytic component has disappeared; at its site, an inflammatory erythematous reaction has occurred.

DISCUSSION

M. Chase: Dr. Klein, I was very interested in the lupus vulgaris with the squamous cell. I would like to know over how long a period the resolution occurred.

E. Klein: For approximately 8 months, with daily administration of amounts of up to 10 cc of second-strength purified protein derivative (PPD) infiltrated in and around the squamous cell carcinoma.

Chase: Did you find any decrease in sensitivity during this period and have to raise the PPD?

Klein: That is why we went up to 10 cc. We started with 1. Yes, we did find a decrease. In fact, we started at Dr. Milgrom's suggestion, and following my discussion with you, and gave her a little time to recover, during which interval we treated her with dinitrochlorobenzene. We did continue challenging her.

W. D. Terry: In view of a number of things that will be discussed during the day, it is important to distinguish between what can be done and maybe what should be done at the present time as we think about clinical application. Of particular interest in this regard are some of the statistical data Dr. Klein hurriedly presented.

You must help us in the area that you have been dealing with, in terms of skin cancers, to put some limits on the kinds of things about which you are talking and to distinguish between what can be done in particular circumstances and what you would opt to do as a physician.

For example, the first patient had a squamous cell carcinoma occurring in a particular circumstance. This carcinoma was rather peculiar in that it hadn't metastasized and had been present for a long time.

Now, from our previous discussions, my understanding is that you would be inclined to treat a patient with squamous cell carcinoma by what would be conventional therapy.

Klein: Absolutely.

Terry: So, where conventional therapy would be inappropriate, you might want to take a crack at seeing what you can do with immunotherapy. In squamous cell carcinoma this would not be your first line of approach.

Klein: No, it would not. I quite agree with you, Dr. Terry, that we ought to try to stick as close as we possibly can to what is real, what is hopeful, and what is halfway in between. But at the same time I do not believe that the content of the papers ought to be adjusted to the press or anybody else. The press are benevolent and knowledgeable, and I think, if they have any questions, they will not be too shy to ask the presenter: "What did you really mean?" Certainly I have been asked that question by 20 different people yesterday, and I fully expect to answer them again today. But I quite agree with you that these are anecdotal.

Terry: I'm not talking about the press because they will ask questions. But other people in the audience are entitled to have that kind of information. With regard to nodular basal cells and the frequency of the nodular responses, for example, again, if you see a patient who has a nodular response, is this the appraoch you would choose?

Klein: No. Saving the world from skin cancer was not the object of my presentation. I wanted to get a point across. I could have gone into the minutia of how I would treat an individual lesion, but the purpose of my presentation was to get a general phenomenon across the numbers, as I said from the beginning, were immaterial in this case. I made it clear to the press that I did not want to have this overexpectancy introduced.

Terry: I am not concerned about the press now; but I am concerned about many of us who are involved with worrying about clinical application.

Klein: Then you must have a separate symposium on skin cancer because you can't possibly deal with this subject in 30 minutes.

Terry: All right.

Klein: Are there any general questions of general pertinence? Does anyone have anything to say about the overall approach? Are we all wet? Should we drop immunotherapy? This is what we're talking about.

D. B. Windhorst: Maybe next to Dr. Klein I have seen as much skin disease as anyone here, and I want to play devil's advocate for a minute. Dr. Klein and I talk back and forth, so I feel free to do this.

I believe in the anecdotal approach as a clinician, and I can match anecdote for anecdote about response of lesions.

The point is that, once we get beyond the anecdotal stage, then comes the question of the clinical trial, about which Dr. Klein and I have talked.

But you ended by emphasizing the animal work, and the general philosophic question that I wanted to raise is: If we have to make a choice as to where we go from here because of funds, etc., which way do we go? Do we try to establish, with appropriate randomized clinical trials, some of the anecdotal data? Or do we dig in really deep with animals that everyone knows are not like human beings?

We must consider this as we talk about anecdotal clinical things.

Klein: We are not the National Cancer Advisory Board. We ought to make some recommendations to them. The amount of money spent on tumor immunology is minuscule compared to that spent in other areas. The important thing is to make more funds and facilities available so we can do whatever we need to do.

Windhorst: I wasn't trying to lead you into that.

Klein: I don't know how else to answer it. Animals and test tube experiments are terribly important, but obviously people are much more important.

Windhorst: I agree.

Klein: Now, how can we help people is the eternal question.

P. Alexander: You showed these skin treatments you have been doing for the last 10 years. At what stage do

you think the time will come to make a proper assessment of their role in therapy (i.e., by comparing your method with the best alternative method and really deciding what their role is)? When can one leave Phase 1 and move to the situation where and when the phenomenon is useful?

Klein: We should have done it 5 years ago to see whether this is better than other available modalities.

BCG in Cancer Immunotherapy: Experimental and Clinical Trials of Its Use in Treatment of Leukemia Minimal and or Residual Disease[1]

G. Mathé, R. Weiner,[2] P. Pouillart, L. Schwarzenberg, C. Jasmin, M. Schneider, M. Hayat, J. L. Amiel, F. De Vassal, and C. Rosenfeld, *Unité Fred-Siguier de Développement Thérapeutique, Institut de Cancérologie et d'Immunogénétique,*[3] *Hôpital Paul-Brousse,*[4] *and Service d'Hématologie de l'Institut Gustave-Roussy,*[5] *94-Villejuif, France*

SUMMARY—Our experimental models of active immunotherapy (AI) of leukemia involved immune stimulation after the establishment of disease in subcutaneously grafted L1210, intravenously grafted Rauscher, and E♀KI leukemia and spontaneous AKR leukemia. We demonstrated that AI can induce tumor regression and produce cures in some animals. Also, we showed that AI is therapeutic if the tumor cell load is $< 10^5$ cells at the onset of AI. This effective threshold can be obtained by grafting 10^5 cells initially or by reducing a higher number of grafted cells by chemotherapy. We applied these criteria derived from animal models to man in our treatment of acute lymphoid leukemia (ALL). The tumor cell load was decreased to the point of minimal residual disease by induction chemotherapy; it was further decreased by cell-reducing, complementary, systemic chemotherapy combined with chemoradiotherapy in the central nervous system. We present the up-to-date result of our first trial started in 1964: Of 20 patients given AI, 7 are still in remission without having had any relapse, after periods varying from 4½ to about 7 years. We also present the overall results of a retrospective study involving 100 patients treated between 1964 and 1971 for whom the pretreatment bone marrow smears were available for review and classification in the 4 varieties of ALL we recently described: prolymphoblastic, macrolymphoblastic, microlymphoblastic, and prolymphocytic. For the microlymphoblastic and prolymphocytic varieties, the actuarial curves for duration of the first remission reached a plateau at 62% and 51%, respectively, for patients < 15 years old and 57% and 46%, respec-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Presented in the absence of Professor G. Mathé by R. Weiner, M.D., who is on fellowship year at Institut de Cancérologie et d'Immunogénétique (on leave from the Department of Medicine, University of California, San Francisco Medical Center, San Francisco, Calif. 94122).

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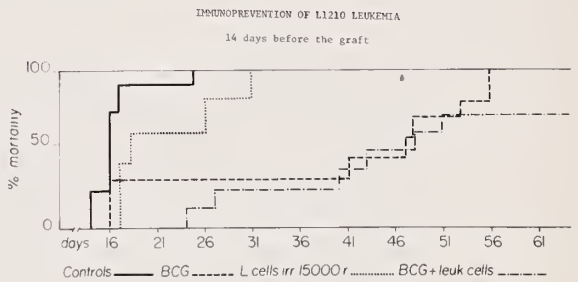
tively, for all patients studied. The actuarial curves of survival also reached a plateau for the macrolymphoblastic variety. We interpret this difference between the curves of duration of the first remission and of survival to indicate the relative sensitivity of the microlymphoblastic and prolymphocytic varieties to immunotherapy and the sensitivity of the macrolymphoblastic variety to only chemotherapy. The prolymphoblastic variety, the curves of which had no plateau, was poorly sensitive to both forms of therapy. We discuss these results in terms of indications for therapy of ALL. The high risks of long-term, intensive, "maintenance" chemotherapy seem justifiable only in the patients with the macrolymphoblastic and prolymphoblastic varieties, whereas the patients with the microlymphoblastic and prolymphocytic varieties should be treated with chemotherapy moderate in intensity and duration before AI.—Natl Cancer Inst Monogr 39: 165–175, 1973.

WHILE THE concept of active *immunoprevention* of cancer has been extensively explored in experimental systems, its clinical implications are remote because, strictly speaking, immunoprevention or prophylaxis demands that no tumor (perceptible or imperceptible) be present at the time of its application. Nevertheless, immunoprevention models have illustrated the potential for use of host responses against malignant cells before they are inoculated. Specific immunostimulation consists of the administration of irradiated neoplastic cells and has generally produced a moderate effect (*1, 2*). Nonspecific immunostimulation used in prevention consists of the administration of one or more systemic immune adjuvants (SIA's), of which BCG has been the most widely used (*2–6*), and has generally produced a marked effect. The combination of specific and nonspecific immunostimulation, even when administered by different routes or at different sites, usually is much more effective than the administration of irradiated tumor cells (ITC) (text-fig. 1).



TEXT-FIGURE 1.—Cumulative mortality of mice grafted with L1210 leukemia, not treated or treated with BCG (first injection 14 days *before* the graft and injections repeated each 4 days) or irradiated leukemic cells (1 injection 14 days before the graft), or combination of both.

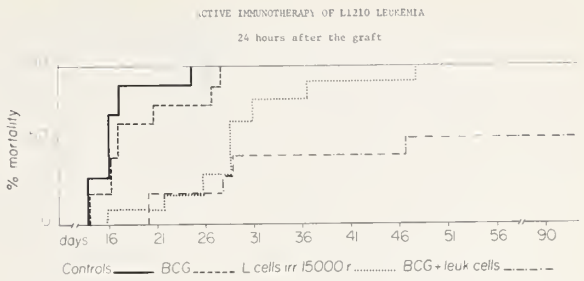
Our current efforts are directed toward effective active *immunotherapy* (AI), clinically applicable in the patient with an established tumor (perceptible or imperceptible). The exploration of immunotherapy as an antitumor weapon stemmed from an urgent need for treatment modalities complementary to chemotherapy which, if one follows first-order kinetics, is theoretically incapable of eradicating "the last cell" (*7–9*). We shall review briefly several animal models which we have studied over the years and from which we have learned some important effects and limitations of active nonspecific (systemic adjuvants), active specific (ITC), and active combination immunotherapy.

L1210 leukemia grafted subcutaneously regressed in response to AI (*2, 10*). This model illustrates several important points: *a*) BCG, when used as an SIA given alone, is rarely and then only slightly effective, while ITC are more actively effective. SIA can potentiate the effect of ITC (even when they are administered at different sites and at different times) so that cures can be obtained (text-fig. 2); *b*) BCG acts only when given repeatedly, whereas ITC given repeatedly manifests no advantage over ITC given once; *c*) AI is effective only if the

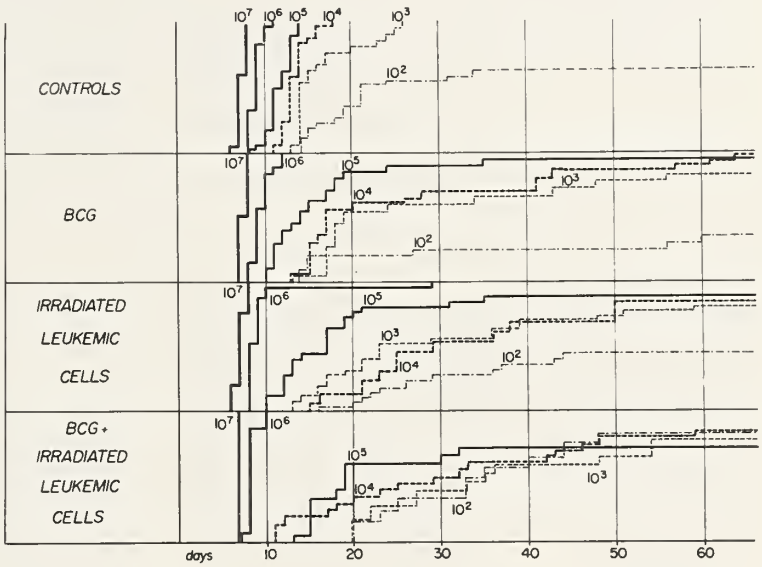
number of malignant cells is low. SIA or ITC, or both combined, were effective only when the total grafted tumor cells was $\leq 10^5$ (text-fig. 3) (*10*). Using similar protocols, we demonstrated the effectiveness of AI in the Rauscher and E♂K1 leukemias grafted intravenously (*11*). In the L1210 (*3*) and E♂G2 (*12*) models, in which chemotherapy was used to reduce the leukemic cell load before immunotherapy, we showed that AI was effective when the grafted tumor load was $> 10^5$ cells, but reduced by noncurative chemotherapy (text-fig. 4).

With the destruction of the myth of tolerance to their leukemia in AKR mice, this spontaneous leukemia became a possible model for immunotherapy. With Doré and Ajuria (*13*), we showed that, after specific immune stimulation, AKR mice can reject an isogeneic graft of the Gross virus-induced tumor (text-figs. 5A and B) and can produce antileukemic cell antibodies. Oldstone et al. (*14*) confirmed this observation. That immune mechanisms play a role in the evolution of spontaneous leukemia in AKR mice has also been demonstrated by Allison (*15*) who showed that immunodepression by administration of antilymphocyte serum shortened the latency period for the onset of spontaneous leukemia. We applied AI to the spontaneous leukemia of AKR mice at 6 months of age, when leukemia is detectable macroscopically at autopsy in 40% of the animals. We took this to be the equivalent of a low tumor-cell load. We have shown that AI in the form of administration of BCG and irradiation- or neuraminidase-treated allogeneic leukemic cells can significantly reduce mortality (text-fig. 6) (*16*).

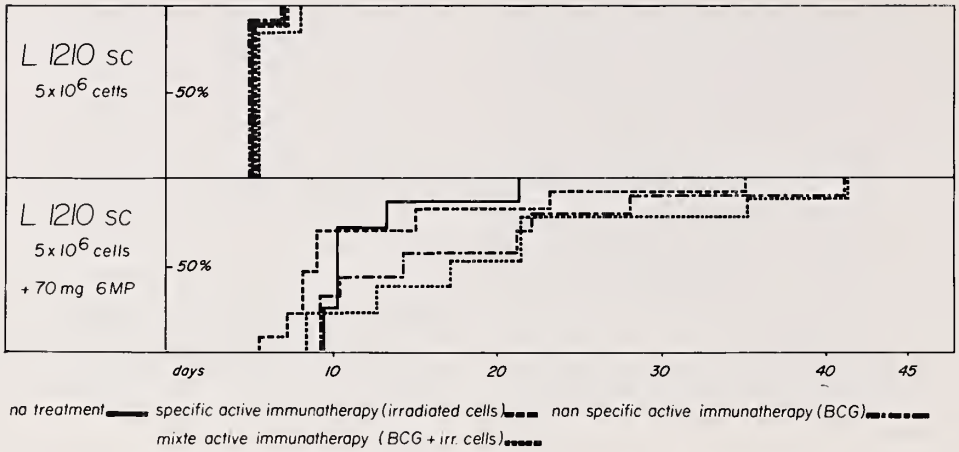
Clearly, in several animal models of leukemia, both spontaneous and transplanted, AI, consisting of SIA's and irradiated allogeneic leukemic cells, can be therapeutic when applied to *minimal disease,* such as the *residual disease after chemotherapy.* We choose acute lymphoid leukemia (ALL) as the first human immunotherapy model on the basis of a scientific reason. Doré



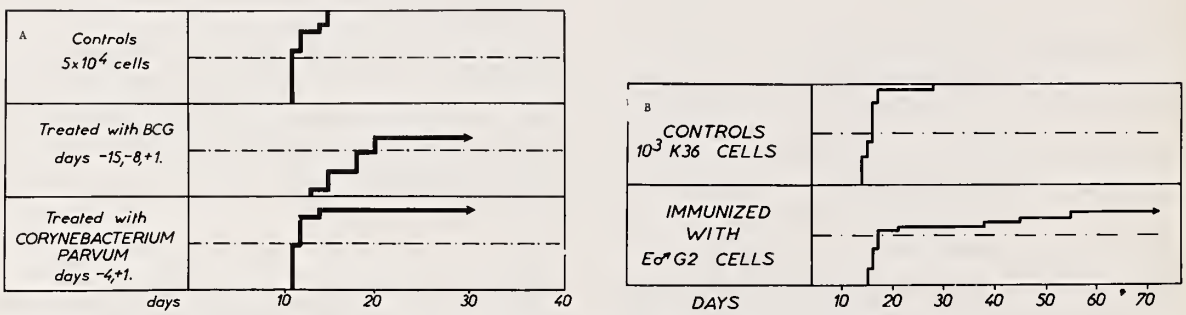
TEXT-FIGURE 2.—Cumulative mortality of mice grafted with L1210 leukemia and not treated or treated with BCG (first injection 24 hr *after* the graft and injections repeated each 4 days) or irradiated leukemic cells (1 injection 24 hr after the graft), or combination of both (*2*).



TEXT-FIGURE 3.—Cumulative mortality of mice grafted with 10^2–10^7 L1210 leukemia cells, not treated or treated, in the 24 hours following the graft, with BCG (repeated injections) or irradiated leukemic cells (1 injection), or combination of both (*10*).



TEXT-FIGURE 4.—AI with BCG and/or ITC (same conditions of application as in the experiment shown in text-fig. 2) applied in mice carrying 5×10^6 tumor cells not reduced or reduced by chemotherapy (70 mg of 6-mercaptopurine) before immunotherapy [Pouillart and Mathé; *see* (*3*)].

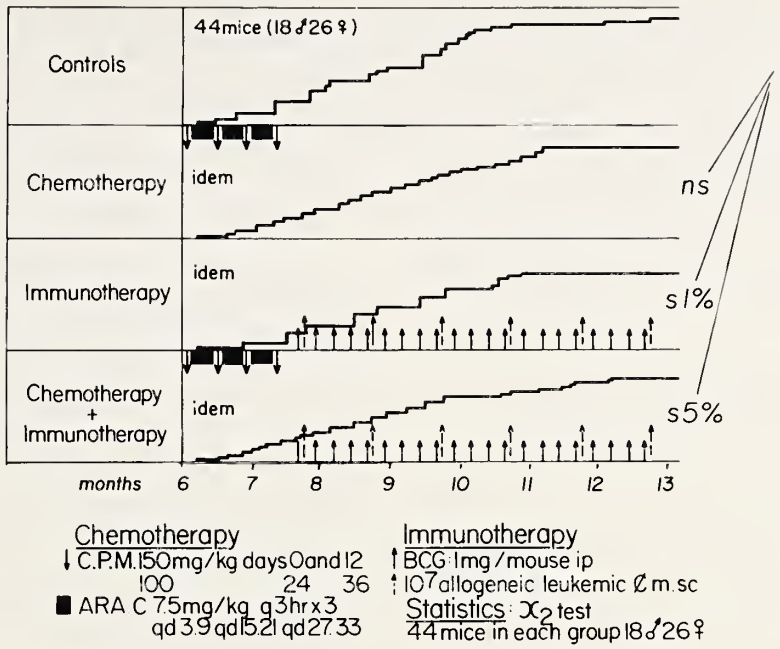


TEXT-FIGURE 5.—Cumulative mortality of 2-month-old normal AKR mice. A) Mice treated with BCG or *Corynebacterium parvum* and then given 10^4 K36 cells intraperitoneally. B) Mice immunized with E♂G2 and then grafted with 10^3 K36 cells.

et al. (*17*) and later Yoshida and Imai (*18*) found immune reaction in some patients with ALL in the form of autologous antibodies against leukemic cells in the serum. More recently, immune reactivity against tumor-associated antigens in patients with ALL was demonstrated in vitro by transformation of the patients' lymphocytes by their leukemic cells (*19*) and by the toxicity of the patients' lymphocytes for their leukemic cells (*20*).

In addition, we chose ALL for the first human trial because it is sensitive to chemotherapy—employing chemotherapy probably cannot be curative (*21*)—and the experimentally demonstrable facilitation of leukemia by immune manipulation is rare compared to that seen in solid tumors (*22*).

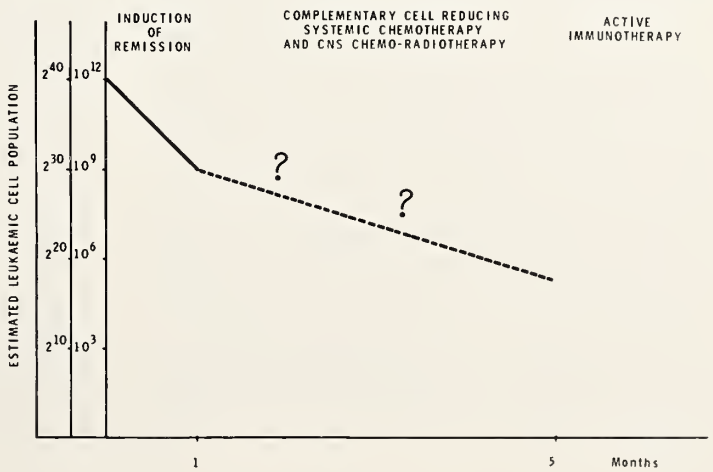
In choosing ALL for the first AI trials, we relied on our animal experiments cited above to suggest the optimum condition for the effi-



TEXT-FIGURE 6.—Cumulative mortality of the different groups of mice according to their treatment.

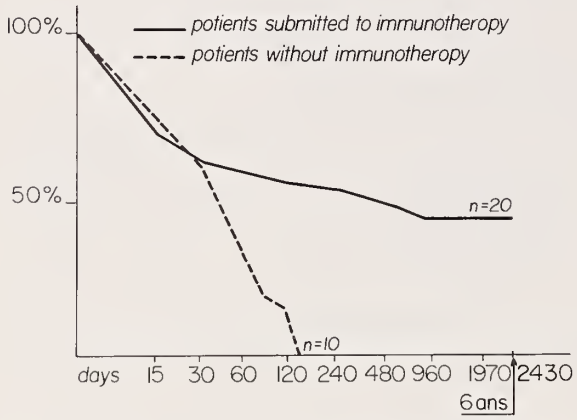
ciency of the trial; i.e., the patient, before starting immunotherapy, should carry the smallest possible number of leukemia cells. To achieve this condition, we reduced the cell number by chemotherapy and induced an apparently complete remission. We then tried to further reduce tumor cell load by sequential complementary systemic chemotherapy. In addition, we gave intrathecal chemotherapy and central nervous system irradiation (text-fig. 7) because of the high incidence of meningeal relapses in ALL patients and the well-known isolation of the central nervous system from systemic immune reactions (*23*). [Our available clinical data confirm the efficacy of both intrathecal chemotherapy and central nervous system irradiation (*24*).] Finally, we administered AI consisting of BCG and/or ITC. BCG (fresh) was provided by the Institut Pasteur.

In a first trial controlled by statisticians, the



TEXT-FIGURE 7.—Principle of the treatment protocols.

protocol of which has been published (25), 20 randomized patients were submitted to AI and compared to 10 randomized controls untreated after complementary chemoradiotherapy. While all the untreated patients had relapses within 130 days after chemoradiotherapy was stopped, 7 of the 20 patients submitted to AI have not yet had relapses, the follow-up after the end of the complementary radiochemotherapy being from 4½ to about 7 years (text-fig. 8).



TEXT-FIGURE 8.—Actuarial curves of patients submitted or not submitted to AI after chemotherapy was stopped. *Note* time scale is geometric.

Other trials have been conducted in which all patients were treated according to the same therapeutic principle. But the protocols differed. They have evolved from 1964, when we started this research, to now, in order to give our patients the benefit of new drugs and their most efficient methods of administration. Text-figure 9 shows that there have been 2 kinds of protocols for chemotherapy in a group of 100 patients. In protocol 4, chemotherapy was short (2½ months) and intensive. It consisted of repeating 3 times the remission induction chemotherapy (prednisone, vincristine, and daunorubicine). In all the other protocols, complementary cell-reducing chemotherapy was longer (5–6 months for protocols 6–8; 7–8 months for protocols 3 and 5; and 24 months for protocol 1); it was less intensive, since only 1 drug (protocols 1, 3, and 5) or 2 drugs (protocols 6–8) were given at a time and at doses adapted to avoid fatal toxicity.

Intrathecal chemotherapy varied from 5 injections of methotrexate (5 mg/injection) to 18 injections of methotrexate combined with cytosine arabinoside (10 mg/injection).[6] Radiotherapy consisted of the application of 1000 rads (protocols 7 and 8) to the whole central nervous system.[7]

AI consisted of "specific" stimulation by ITC and nonspecific stimulation by BCG applications combined for some protocols (#5–8) with injections of *Corynebacterium parvum* or *C. granulosum* and, in the latest trials (#4 and 8), of poly I:C as a third component. *C. parvum* and *C. granulosum* were provided by the Institut Pasteur, and poly I:C was provided by the Laboratoire Choay. We have verified their ability to stimulate immune reactions in our screening system in mice (*22, 26*).

Details on the administration of BCG, *C. parvum, C. granulosum,* poly I:C, and ITC were published recently (*27*).

The patients who have relapses under immunotherapy are retreated according to the complete protocol being evaluated on the service at the time of the relapse; i.e., they are resubmitted to the remission induction chemotherapy, then to the complementary chemoradiotherapy, and then again to AI.

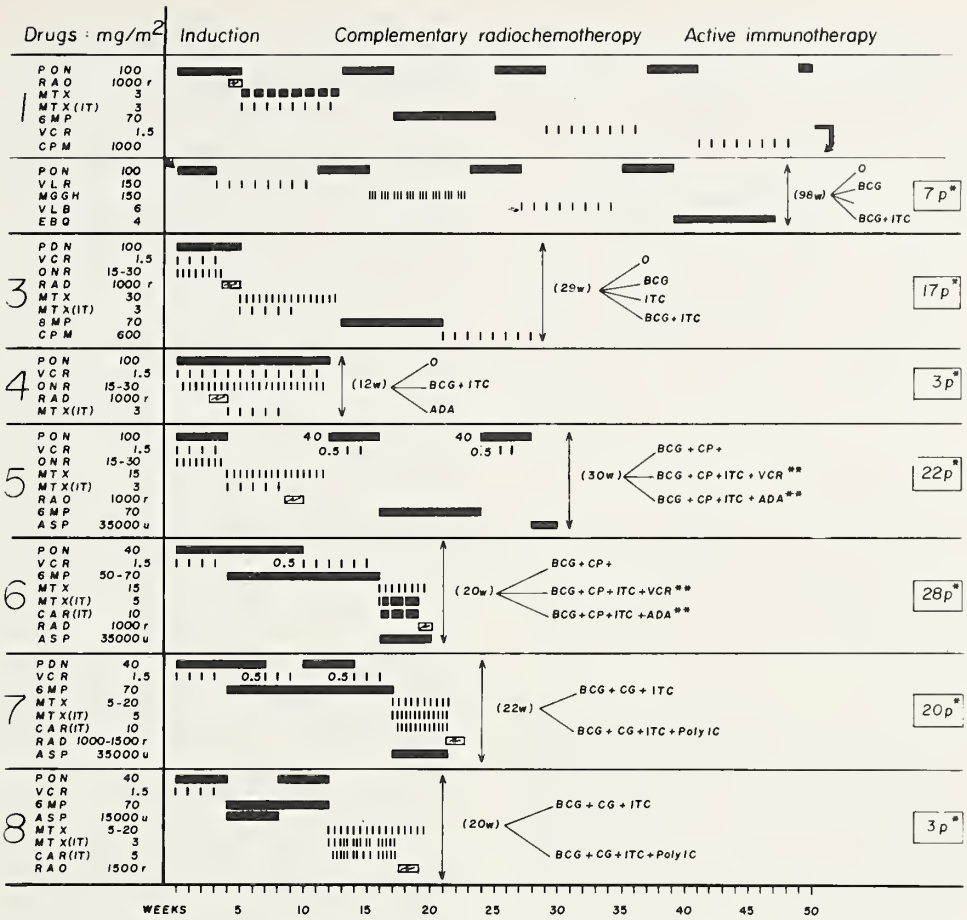
We recently reported the results of our trials in 100 patients selected by the following criteria: *a*) The patients were treated from the onset by our protocol and *b*) a pretreatment bone marrow smear was available for morphologic review.

The results of these trials were reported with the use of actuarial curve of cumulative duration of the first apparently complete remission and of the cumulative duration of survival. All curves were presented with the necessary *geometric time scale.*

Text-figure 10 shows that, for patients of all ages, the median length of the cumulative duration of the first apparently complete remission was 12 months, and the median length of the cumulative duration of survival was 30 months.

[6] Our recent analysis has shown that this combination adds no benefit to methotrexate alone as far as the incidence of meningeal relapses is concerned (*24*).

[7] This radiotherapy adds a significant benefit to intrathecal chemotherapy (*24*).

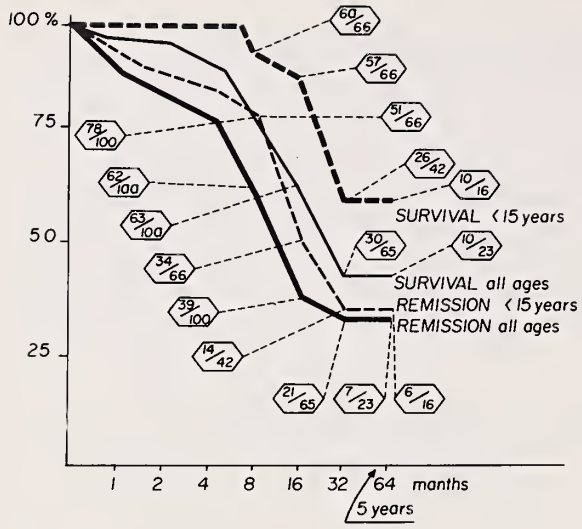


TEXT-FIGURE 9.—The different protocols to which the 100 patients of the present study were submitted. *Number of cases in which diagnostic bone marrow smears were available to be reviewed for this study. **For doses, *see* (*32*). PDN = prednisone; RAD = irradiation; MTX = methotrexate; IT = intrathecal; 6MP = 6-mercaptopurine; VCR = vincristine; CP = *C. parvum*; VLR = vinleurosine; MGGH = methylglyoxal-bis (guanylhydrazone); VLB = vinblastine; EBQ = ethylene-imino-benzo-quinone; DNR = daunorubicine; ASP = L-asparaginase; CAR = cytosine arabinoside; ADA = adamantadine; and poly IC = polyinosinic-polycytidylic acid.

Most interesting is the observation that these curves, after descending, broke at about the 32d month and then reached a *plateau*. The fraction of the patients—the cumulative duration of the first apparently complete remission being represented by this plateau—was 33%. This was the statistical expression of "cure expectancy."

The median length of cumulative duration of the first apparently complete remission of the patients < 15 years old was 16 months, and the fraction of the patients demonstrating the plateau phenomenon was 43% (text-fig. 10). The curve of the cumulative duration of survival has not as yet fallen to the 50% level. The prognosis, therefore, was significantly better in patients < 15 years old than in the total population of patients.

The most interesting observations concern the variations between the cumulative duration of the first apparently complete remission and the cumulative duration of survival according to the cytologic varieties we described in this disease (*28*), which we called prolymphoblastic acute leukemia, macrolymphoblastic acute leukemia, microlymphoblastic acute leukemia, and prolymphocytic acute leukemia (*29, 30*). While the curves for the cumulative duration of apparently complete remission for the patients with prolymphoblastic acute leukemia and those with macrolymphoblastic acute leukemia descended



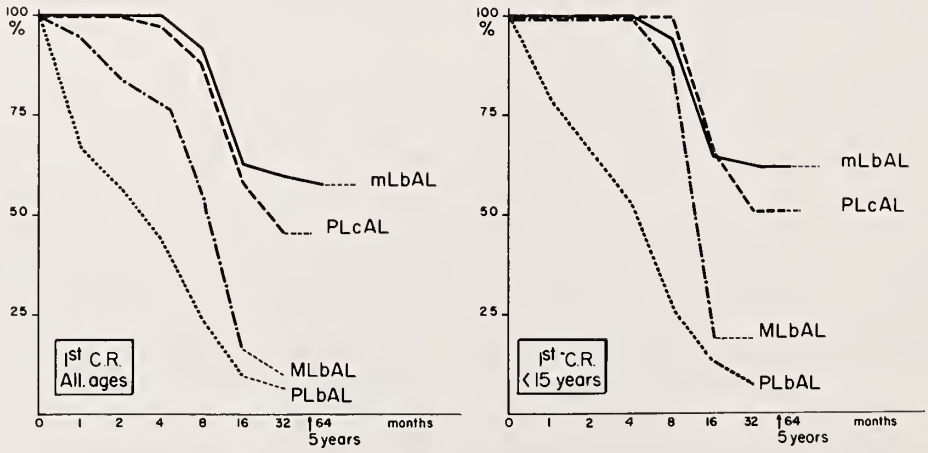
TEXT-FIGURE 10.—Actuarial curves of duration of first apparently complete remission and of length of survival for all ages and for patients <15 years old. *Note* time scale is geometric.

regularly to a very low percentage at 16 months, those of the patients with microlymphoblastic acute leukemia and those with prolymphocytic acute leukemia broke between 16 and 32 months to reach a plateau for about 46% of the patients with prolymphocytic acute leukemia and 57% of those with microlymphoblastic acute leukemia (text-fig. 11). Text-figure 11 also shows that this plateau was slightly higher in patients < 15 years old (62% for the microlymphoblastic type) than in the total patient population.

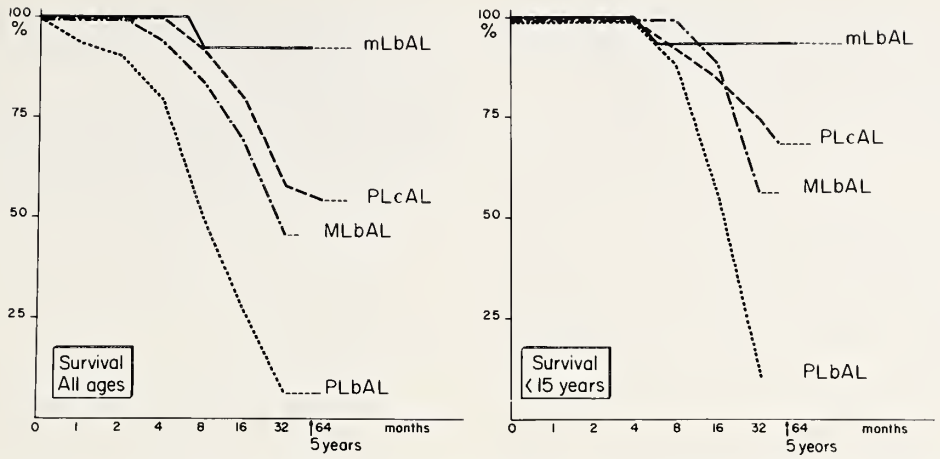
An unexpected observation is presented in text-figure 12 which shows the actuarial curves of the cumulative duration of survival. For the patients of all ages and for the patients < 15 years old, there was a plateau representing about 50% of the population, not only for the 2 preceding varieties but also for the macrolymphoblastic variety. This difference between the cumulative duration of the first apparently complete remission under immunotherapy and the cumulative duration of survival suggests that while the microlymphoblastic and the prolymphocytic types are immunotherapy sensitive, the macrolymphoblastic type is immunotherapy insensitive but chemotherapy sensitive. This interpretation is supported by the fact that, according to our protocol, a patient who has a relapse under immunotherapy is resubmitted to to remission induction chemotherapy, which is frequently successful when followed by preimmunotherapy chemotherapy and then immunotherapy.

The following facts are evident from the data we have accumulated over the past 8 years.

1) A fraction of the ALL patients (33% for subjects of all ages and 43% for those < 15 yr old) are still in apparently complete remission. The curve for the cumulative duration of apparently complete remission for these patients



TEXT-FIGURE 11.—Comparative cumulative total duration of first remission with the different cytologic variety of ALL: all ages and patients < 15 years old (actuarial curves). *Note* time scale is geometric. mLbAL = microlymphoblastic; PLcAL = prolymphocytic; MLbAL = macrolymphoblastic; and PLbAL = prolymphoblastic.



TEXT-FIGURE 12.—Comparative cumulative survival of the ALL patients according to cytologic variety of ALL: all ages and patients <15 years old (actuarial curves). *Note* time scale is geometric. mLbAL = microlymphoblastic; PLcAL = prolymphocytic; MLbAL = macrolymphoblastic; and PLbAL = prolymphoblastic.

breaks at about 32 months and continues as a plateau. The distribution of our patients currently receiving immunotherapy is given in text-figure 10. This represents all our patients in remission, not just those included in the study of 100 patients.

2) The existence of the plateau differs noticeably among the cytologic varieties we have proposed. The curves for the cumulative duration of apparently complete remission present a plateau for a high percentage of patients with only 2 cytologic varieties, microlymphoblastic and prolymphocytic. The percentage is especially high for the microlymphoblastic type, which develops more frequently in children. The curve for the cumulative duration of apparently complete remission does not plateau for the macrolymphoblastic variety, while the curve for the cumulative duration of survival for this variety is immunotherapy insensitive but chemotherapy sensitive reaches a plateau at 16 months.

We have demonstrated over the last 8 years that ALL can be treated by immunotherapy. We have used protocols based on our successful animal models and have adhered to the precepts derived from those models. 1) We reduce by chemotherapy the leukemic cell load to as low a level as possible to avoid the toxicity caused by prolonged immunosuppression. 2) We apply nonspecific immune stimulation using fresh, live BCG as our basic SIA and administer the BCG at frequent intervals by scarification. We have investigated other SIA's at various times as well, always in addition to, but never substituting them for BCG. 3) We apply specific immune stimulation in the form of irradiated allogeneic leukemia cells by the intradermal and subcutaneous routes. 4) We apply special treatment to the central nervous system before immunotherapy because of the isolation of the central nervous system from systemic immune responses.

Our patients under immunotherapy show no clinical evidence of susceptibility to the disease of immunosuppression. By contrast, the accidents and deaths attributable to vigorous so-called "maintenance" chemotherapy are not rare, being 16% in some reports (*31*) and more in another (Glidewell and Miescher, personal communication). Hence, we believe that the cytologic correlation is potentially important for determining prospectively the therapeutic protocols.

The choice between protocols comprising moderate complementary chemotherapy followed by immunotherapy and protocols comprising intensive and/or long-term chemotherapy must no longer be determined by the geography of the patients or their doctors, but by the cytologic variety of the patients' disease. While it seems sound to treat the microlymphoblastic and prolymphocytic types with moderate chemotherapy followed by immunotherapy, it seems reasonable

to risk a more intensive and longer chemotherapy only for the other types, prolymphoblastic and, especially, macrolymphoblastic.

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Immunotherapy in Previously Treated Acute Lymphatic Leukemia[1, 2]

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SUMMARY—A total of 25 patients with previously treated acute lymphatic leukemia were given asparaginase either alone or combined with actinomycin D to induce remission. Of them, 16 who achieved remission were randomly assigned to maintenance therapy with either weekly BCG plus allogeneic leukemia cells (9 patients) or methotrexate (MTX) in 5-day pulses, plus allogeneic cells (7 patients). Remission duration was equivalent in both groups, 90 and 150 days, respectively; there was no evidence of enhancement with BCG administration. Specific sensitization to tuberculin occurred both in vivo and in vitro in the BCG-treated group. Skin testing revealed an increase in reactivity to recall antigens (candidin, steptokinase-streptodornase, and mumps) in both groups. In vitro, the MTX-treated group showed a cyclic increase in response to antigens after each course of MTX. The BCG-treated group showed a significant rise in response to only smallpox antigen. The response to immunizing cells followed the same pattern in vivo and in vitro as the response to recall antigens. Those patients with positive skin reactivity to immunizing cell membrane preparations or vigorous in vitro blastogenic response to autologous leukemia cells had longer remissions than those who were nonreactive. These data suggest some increase in immune reactivity in general in both groups of patients and an association between reactivity to leukemia antigen and a good prognosis for remission duration even in a patient with relatively advanced disease.—Natl Cancer Inst Monogr 39: 177–187, 1973.

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[4] National Institutes of Health, Public Service, U.S. Department of Health, Education, and Welfare.

RECENTLY, interest has been stimulated in the possibility of immunotherapy in human leukemia. Prolonged remission duration was seen after maintenance treatment of acute leukemia patients with injections of pooled allogeneic leukemic cells and living BCG administered by scarification (*1*). Analysis of these results suggested that BCG acts as an adjuvant that increases the reaction to possible leukemia antigen. To investigate this possibility, we compared the effects of immunization with allogeneic leukemia cells combined with either BCG or methotrexate (MTX). We assessed the effect on remission duration as well as on lymphocyte response in vivo and in vitro to immunizing cells and to other antigens.

MATERIALS AND METHODS

The study began with 25 patients. All patients had acute lymphatic leukemia and had been previously treated at the National Institutes of Health for a median of 23 months (range 1–88). They were either resistant to remission induction with vincristine and prednisone or had had at least 2 relapses.

Most patients were children; only 3 were >21 years old. The median age was 10 years (range 1–58). There were 7 females and 18 males. Remission duration was measured from the date bone marrow remission was achieved (defined as <5% leukemic blasts in the marrow), to the date marrow relapse occurred.

Induction Therapy

Initially, patients were randomly assigned to remission induction will either asparaginase (*Escherichia coli*, Merck, Sharp & Dohme) given alone at 15,000 IU/m²/day for 28 days (10 patients) or asparaginase plus actinomycin D (Merck, Sharp & Dohme) at 0.4 mg/m²/day for 5 days on days 1–5 and 15–20 of asparaginase therapy (12 patients).

In addition, 3 patients were entered on the maintenance protocol, after remission had been induced with asparaginase plus Cytoxan at 500 mg/m² on days 5, 7, 13, and 14. In 2 patients who developed asparaginase sensitivity, *Erwinia carotovorum* enzyme from Porton Laboratory was used.

Maintenance

Cells.—Leukemia blast cells were obtained by leukopheresis from patients on admission. They were stored in 10% dimethyl sulfoxide in liquid nitrogen at −180° C until they were ready for use, at which time they were thawed rapidly to 37° C and washed once (*2*). Each injection was prepared with 4×10^7 cells in 0.1–0.2 ml. A single allogeneic donor was used for each patient.

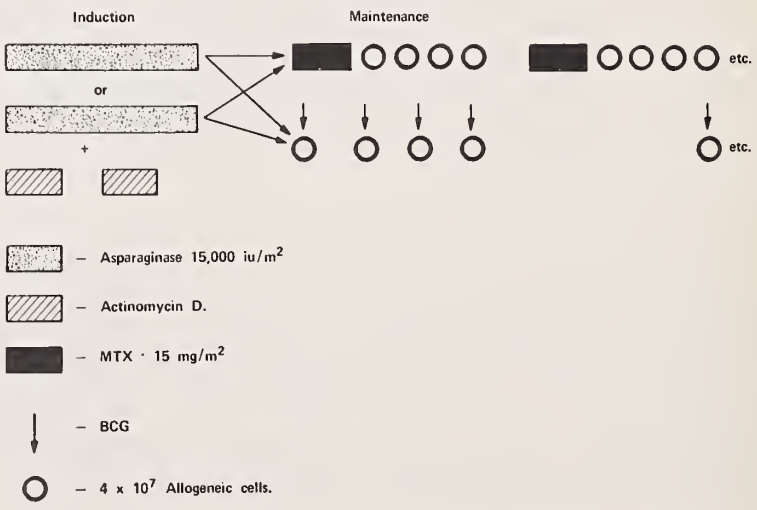
MTX.—Obtained from the Lederle Laboratories, MTX was given in 5-day courses at 15 mg/m²/day every 30 days. Cells were given at 8, 10, 12, and 14 days after MTX administration.

BCG.—Obtained fresh from the Pasteur Institute every 2 weeks, BCG was given by scarification, with approximately 15 scarifications in a 3-cm² area; 0.5–1.0 cc of fresh BCG was applied and allowed to dry. Cells were given intradermally (id) somewhere between the BCG lesion and draining lymph nodes. BCG and cells were given weekly for 4 weeks and every 4th week thereafter.

The general scheme for the protocol is shown in text-figure 1.

Immune Evaluation

Skin tests.—Membrane extracts of autologous and al-



Text-figure 1.—General scheme of treatment protocol.

logeneic leukemia cells were prepared after disruption of the cells by freezing and thawing as previously described (3). Skin tests were performed by id injection of 0.1 ml membrane preparation, usually on the patient's back. The extracts were tested at a protein concentration of 1 mg/ml. At this concentration, extracts of normal lymphocytes have not elicited positive delayed reactions in any normal individuals (4). Skin tests were performed at the same time with 0.1 ml of each of the following antigens: tuberculin [intermediate strength of purified protein derivative (PPD) from Parke Davis & Co. or intermediate strength PPD (Mantoux). from Connaught Medical Research Laboratories, University of Toronto]; mumps (Eli Lilly & Co.), candidin [dermatophytin "0" (1:100), Hollister-Stier Laboratories]; and Varidase [streptokinase (40 U)-streptodornase (10 U), Lederle Laboratories].

Skin tests were examined at 24 and 48 hours after inoculation and measurements made with a vernier caliper. Induration of ≥ 5 mm in diameter at 48 hours was considered a positive skin test.

In vitro lymphoctye response—Mixed leukocyte cultures (MLC) were performed by a previously reported modification (5) of the method of Bach and Voynow (6). Cultures were set up in triplicate in 12 × 75-mm glass tubes with 2×10^5 responding lymphocytes in 1 cc Eagle's medium (Flow Laboratories) supplemented with 10% normal human serum (Flow Laboratories). Mitomycin C-treated cells were added at a concentration of 5×10^5 in 0.1 ml. Leukemia blast cells used for immunization and cells from normal unrelated individuals were frozen and thawed in the same way, so that comparable preparations were available.

Stimulants (0.1 ml) were added to other triplicate sets of cultures as follows: phytohemagglutinin P (PHA) (Difco Laboratories), 1:60 dilution from a reconstituted 5-ml vial; lyophilized PPD (Lederle Lahoratories), 10 μg; streptolysin O reagent (SLO) (Difco Laboratories), with standard dilution of vial to 25 ml; smallpox vaccine (Wyeth Laboratories), contents of a single vial diluted in 30 cc medium; and Candida [*Monilia albicans* allergenic extract 20,000 PNU/cc (Hollister-Stier Laboratories)], 1:10 dilution

The amount of blastogenesis was assessed by ^{3}H-thymidine incorporation (5). Cultures stimulated with PHA were harvested after 3 days of incubation. MLC and cultures stimulated with antigen were harvested after 6 and 7 days of incubation. Results are expressed as stimulation ratios, i.e., $\frac{\text{dpm in stimulated cultures}}{\text{dpm in control cultures}}$

Immunoglobulin levels.—Quantitative immunoglobulin levels were measured by radial diffusion in agar gel (7). Values for normal individuals in this laboratory have been previously reported (8).

HL-A Typing and Antibody

HL-A typing of immunizing blast cells and screening of patients' serum for anti-HL-A antibodies after immunization were performed by the method of Mittal et al. (9).

RESULTS

Induction and Duration of Remission

There was no apparent difference between the 2 induction regimens: Remission was achieved in 7 of 10 patients treatd with asparaginase alone and in 8 of 10 who received an adequate trial with the asparaginase-actinomycin D combination. Where actinomycin D was withheld for 2 patients originally assigned to combination therapy, the combination trial was considered inadequate. Of those entering remission, 1 patient refused maintenance and 1 died of peumonia while in remission. With the 3 patients in remission after asparaginase induction, although not randomly assigned to that treatment, a total of 16 patients were available for maintenance assignment. Data from the patients in each maintenance group were pooled and considered together: The 7 patients who received MTX and cells had a median remission duration of 151 days (range 41–305); the 9 who received BCG and cells had a median remission duration of 91 days (range 44–231). There was no significant difference between the 2 groups. Three patients, two on BCG arm and one on MTX arm, developed an initial bout of meningeal leukemia while on this protocol. Two others had relapses of previously diagnosed meningeal leukemia.

Toxicity

Major toxicity observed is shown in table 1. Of the patients randomly assigned to the combination induction, 2 did not receive actinomycin D. For 1 patient, the induction attempt was discontinued after 5 days because of bleeding associated with a severe coagulation protein abnormality. For the other, jaundice and severe gastrointestinal pain associated with asparaginase therapy led us to withhold the actinomycin D. During induction, most patients (19/22) showed depression in white blood cell count, with a total $<3000/mm^3$; for 10 patients, the counts fell to a dangerous level of < 500 granulocytes/mm^3. Alkaline phosphatase was elevated in 15 of the 22 patients to levels 2–3 times the upper limit of normal. In 11 patients, this elevation persisted during maintenance. Abnormalities of the transaminase levels were seen in 4 patients

TABLE 1.—Toxic reaction to remission induction and maintenance therapy

Regimen	Total No. of patients	Total white blood cell count <3000/mm³	Granulocyte count <500/mm³	SGOT* >40	Alkaline† phosphatase >115	Other
Induction						
Asparaginase	10	9	6	1	7	Abnormal prothrombin time, 2 Renal failure, 1 Psychiatric disorder, 1
Asparaginase actinomycin+D	12	10	4	3	8	Abnormal prothrombin time, 1 Severe abdominal pain, 2 Jaundice, 2
Maintenance						
BCG	9	3	0	0	6	
MTX	7	1	1	4	5	

* Serum glutamic oxaloacetic transaminase (Karmen units).
† International units.

on the MTX-maintenance arm and in 2 of them were severe enough to require reduction in dosage. The MTX regimen appeared to be slightly more hepatotoxic than the BCG.

Cell injections were well tolerated, although a few patients complained of generalized malaise after treatment. The procedure of scarification for BCG administration was painful, but once it was complete, healing was rapid with no permanent scar formation. No local adenopathy or systemic symptoms were seen. Itch and induration were present in most of the lesions after about a week. Induration tended to persist longest at the sites where 2 scarification lines crossed each other. In 1 patient, multiple small id injections of BCG and cells were given at sites 0.5 cm from one another. These coalesced to form a large ulcer which had not healed when the patient died 2 months later.

Immune Evaluation

Table 2 shows the results of skin tests in both groups of patients. The most dramatic change was in the response of the BCG-treated group to tuberculin. Of 9 patients, 7 converted from negative to positive. The eighth changed from 0 to 4 mm of induration, and the ninth patient was nonreactive to all skin test antigens. The mean diameter of the response to candidin and Varidase (SKSD) doubled; 4 of 9 patients converted from negative to positive with candidin and 2 of 9 with SKSD; 2 of 9 patients also converted from negative to positive with mumps antigen, but the mean diameter of the skin test induration did not increase. In the MTX-treated group, 1 patient converted from negative to positive with PPD. The group as a whole showed an increase in mean skin test induration diameter from 2.6 to 4.5 mm in response to candidin, 8.4 to 14.7 mm in response to SKSD, and 7.6 to 13.8 mm in response to mumps antigen; 2 of 5 patients converted from negative to positive against Candida, and 1 of 5 against SKSD. One patient was not evaluable because, before therapy, she gave a 55-mm induration response to SKSD which obscured the other skin test responses and made her unwilling to have skin tests repeated. Another patient complained of discomfort on skin testing and therefore received a restricted number of antigens. Both treatment groups showed generalized similar increases in reactivity to all skin test antigens.

The in vivo response to leukemia blast cell membranes is shown in table 3. Negative reactors are included as 0 mm in these calculations. Two of the six patients in the MTX group and three of six evaluable in the BCG group converted their reactions to extract of the immunizing cells from negative to positive and one in each group remained positive during the evaluation. In each group, 2 patients were initially negative in reaction to immunizing blast cells and remained so; 1 patient in the MTX group converted from

TABLE 2.—Skin test reactions to recall antigens

	PPD	Candida	SKSD	Mumps
BCG Treatment Group				
Diameter in mm induration mean (range)				
Before immunotherapy	0 (0–1)	3 (0–18)	8.1 (0–16)	6.6 (0–13)
After immunotherapy	10 (0–24)	5.3 (0–11.5)	16.1 (0–24)	7.6 (1–14)
Reactivity before and after therapy				
Negative to positive	7	4	2	2
Positive to positive	0	1	6	5
Positive to negative	0	1	0	1
Negative to negative	2	3	1	1
Number evaluable	9	9	9	9
MTX Treatment Group				
Diameter in mm induration mean (range)				
Before immunotherapy	0.6 (0–2)	2.6 (0–7)	8.4 (0–19)	7.6 (2–10.5)
After immunotherapy	2.8 (0–6.5)	4.5 (0–9)	14.7 (0–26)	13.8 (6.5–13.5)
Reactivity before and after therapy				
Negative to positive	1	2	1	1
Positive to positive	0	2	3	5
Positive to negative	0	0	0	0
Negative to negative	4	1	1	0
Not tested on 1 or 2 occasions*	2	2	2	1
Number evaluated	7	7	7	7

* *See* text for explanation.

TABLE 3.—Skin test response to cell membranes

	BCG-treated group		MTX-treated group	
	Immunizing	Autologous	Immunizing	Autologous
Diameter in mm induration mean (range)				
Before immunotherapy	1 (0–6)	0 (0)	2.2 (0–5.5)	3.8 (0–5.5)
After immunotherapy	3 (2–5.5)	4.5 (0–9)	2.7 (0–5.5)	2.5 (0–5)
Reactivity before and after therapy				
Negative to positive	3	1	2	0
Positive to positive	1	0	1	1
Positive to negative	0	0	1	0
Negative to negative	2	1	2	1
Number evaluable	6	2	6	2

positive to negative. The remission duration for the 3 patients in the MTX group who either were or became positive was 126, 225, and 305 days, while those who either were or became negative had remissions of 41, 63, and 151 days. In the BCG group those who were or became positive had remissions of 150, 182, 204, and 231 days, while those who remained negative had remissions of 44 and 91 days. Thus patients with positive reactivity to immunizing cells had a median remission duration of 204 days (range 126–305), while those with negative reactivity had a median remission of 63 days (range 41–151). These results were statistically significant at the 0.01 level by the two-tailed rank sum test of Wilcoxon (*10*).

Because only 2 patients in each group could be tested for reactivity to autologous blast cell

material, no conclusions were made about its correlation with clinical state.

In Vitro Response to PHA and Antigens

Table 4 shows the response of patient's lymphocytes in vitro to PHA and 4 antigens. Data from normal controls whose cells were studied on the same days as the patients' are included. The results for the MTX-treated patients are expressed in weeks after the 1st day of MTX. This means that the value designated week 1 resulted from study done on blood drawn 2 days after the last MTX dose. The MTX-treated patients had normal reactivity to PHA before therapy. This reactivity fell slightly (*73*) with MTX administration and then rose to a peak higher than the original level (408) after 4 weeks. Similar "rebound" in immunologic response after therapy to values higher than those seen before therapy occurred in response to SLO, smallpox, and candidin at 2 weeks after administration. This type of cyclic increase in immune response occurred repeatedly with each course of MTX. The response profile of 1 patient on MTX who stayed in remission for several months and showed this cyclic pattern is shown in text-figure 2. The response to PPD and PHA did not show a consistent change.

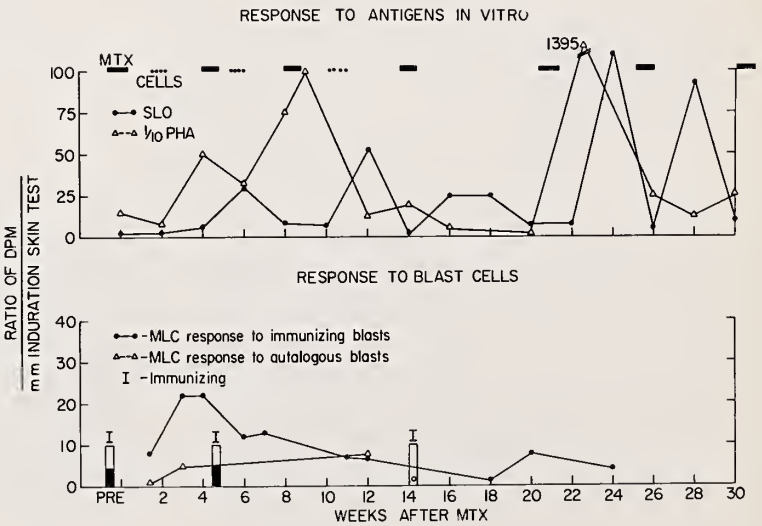
Table 4 also shows that the BCG-treated patients initially had a PHA response ratio of 255. This ratio fell to 149 with BCG administration and remained at that level over the next 4 months. PPD response began to increase after 4 weeks from a baseline of 6.1 to a stimulated value of 21. This response remained elevated. In several patients treated with BCG, there was a fall in in vitro reactivity to recall antigens during the weeks the BCG was administered, which then returned to normal levels by week 6. After this, the response to SLO and candidin showed no consistent change in these patients; but their response to smallpox vaccine rose progressively throughout the study period and the final level of 4.7 is 6 times the baseline ratio of 0.8.

Text-figure 3 details the in vitro response of 1 patient receiving BCG and cells.

Mixed Leukocyte Culture

Response to Immunizing Cells

Table 5 shows the responses in MLC to immunizing cells. In general, frozen blast cells did not stimulate as well in MLC as frozen fresh normal lymphocytes. Lymphocytes from healthy unrelated individuals, cultured with the blasts used to immunize the BCG-treated patients, showed a median response ratio of 4.5 (range 1.0–476). The response of unrelated individuals to the blasts used to immunize the MTX-treated patients was somewhat higher at 12.5 (range 0.6–354). This same pattern was seen in the patients, with the effect of immunization superim-

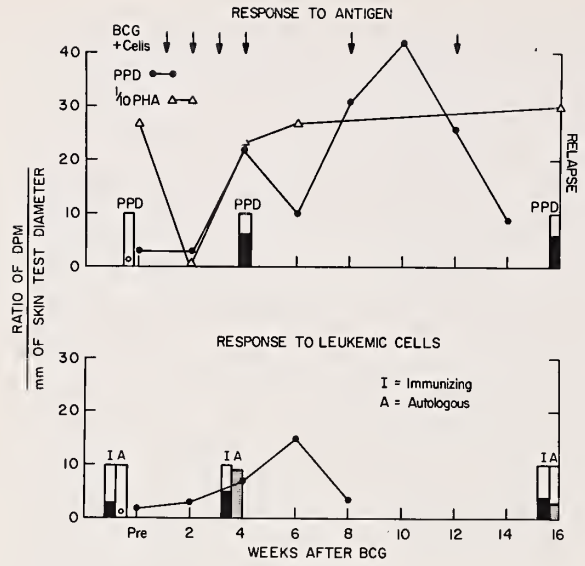


TEXT-FIGURE 2.—Response of a patient on MTX to antigens and blast cells. *Top panel:* Rebound in response to antigen in vitro with repeated courses of MTX can be seen. *Bottom panel:* There is a transient increase in response to immunizing cells in vivo and in vitro as well as an increase in response to autologous cells.

TABLE 4.—Response of lymphocytes in vitro to PHA and antigens*

		MTX-treated patients (wk after MTX)						BCG-treated patients (wk after BCG)					
	Control	Pre-	1	2	3	4	5	Pre-	1–2	3–4	5–8	9–14	≥15
PHA	160 (21–2267)†	118 (29–130)	73 (15–1205)	157 (5–537)	131 (28–1787)	408 (81–1010)	161 (62–436)	255 (58–331)	149 (29–1325)	130 (2–707)	115 (57–430)	121 (27–469)	116 (41–516)
PPD	5.6 (0.8–226)	3.8 (0.8–22)	2.5 (0.9–43.8)	4.4 (1.0–23)	1.4 (0.3–10)	2.5 (0.7–24)	3.0 (2.0–6.8)	6.1 (0.5–35)	5.4 (1.7–26)	3.5 (0.8–100)	21 (2.7–153)	14.4 (3.0–42)	19.5 (9–47)
SLO	12.5 (0.8–309)	0.6 (0.2–12.7)	5.6 (0.4–140)	11.8 (0.3–146)	7.1 (0.2–52)	5.1 (0.3–160)	11.4 (0.8–181)	5.8 (0.6–246)	4.9 (0.4–32)	3.4 (0.4–100)	7.1 (0.5–22)	2.0 (0.3–55.2)	6.2 (0.1–39)
Smallpox	1.6 (0.2–42.3)	3.5 (0.4–103)	2.4 (0.8–78)	6.2 (0.5–31)	1.5 (0.3–13)	3.0 (0.4–83)	10 (0.5–16)	0.8 (0.4–5.8)	1.3 (0.4–19)	0.8 (0.2–14.2)	1.4 (0.2–31)	3.0 (0.2–18.7)	4.7 (0.5–54.5)
Candidin	5.8 (0.9–78.3)	4.8 (1.4–99)	4.5 (0.7–53.5)	19.6 (0.8–56)	9.3 (1.1–32)	5.9 (1.0–265)	8.7 (3.2–108)	3.8 (0.8–534)	4.2 (1.0–92)	3.2 (1–42)	6.9 (0.5–590)	4.0 (0.8–84)	3.2 (0.5–162)

* Expressed as median stimulation ratios (*see* "Materials and Methods").
† Numbers in parentheses denote range.



TEXT-FIGURE 3.—Response of a patient on BCG to antigen and leukemic cells. *Top panel:* There is a specific increase in the response to PPD in vivo and in vitro. PHA response shows an initial drop with active BCG therapy and subsequent return to normal levels. *Bottom panel:* A transient in vitro response to immunizing cells is seen with a more persistent in vivo response to both immunizing and autologous cells.

posed. Table 5 shows that, before immunization, the BCG-treated patients had a median response of 4.0 to immunizing cells, which tripled to a ratio of 11 by 3–4 weeks after immunization and remained at 9 after 5 weeks. The MTX-treated patients showed the rebound pattern with a pretreatment ratio of 9 in response to immunizing cells, which doubled to 21 by 3–4 weeks after immunization and returned to baseline levels after 5 weeks.

Table 5 also shows the response of the same patients to cells (frozen and thawed in the same manner as the blast cells) from an unrelated healthy individual. The median ratio of unrelated normal controls reacting to these normal cells was 26.4. Before therapy, the BCG-treated patients had a median response of 12.5 to the normal cells; after 3–4 weeks, this response had risen to 31 and then remained elevated. The MTX-treated patients had a ratio of 20 before therapy, 52 by 3–4 weeks after immunization, and 30 after 5 weeks.

Response to Autologous Cells

Autologous blast cells were available only in a few patients, and MLC response to these cells is shown in table 6. For 3 patients in the BCG group, reactivity was 2.8–8 after 3–8 weeks of therapy. For 3 patients in the MTX group, all of whom had no reaction prior to therapy, stimulation ratios were 1, 4, and 5. Those patients with the longer remission duration also had higher stimulation ratios; however, the members are too small to draw any statistical conclusion.

Immunoglobulin Levels

Table 7 shows the immunoglobulin levels for the 2 groups of patients before and after therapy. There were no significant differences.

TABLE 5.—MLC response*

Cells	Control	Weeks after immunization					
		MTX			BCG		
		Pre-	3–4	5	Pre-	3–4	>5
Immunizing	BCG 4.5 MTX 12.5	9 (0.1–31)†	21 (0.1–125)	8 (0.1–46)	4 (0.1–109)	11 (0.3–84)	9 (2–10)
Allogeneic	26.4 (0.4–400)	20 (4–100)	52 (29–500)	31 (4–110)	12.5 (5–130)	31 (4–190)	29 (2–120)

* Expressed as median stimulation ratios (*see* "Materials and Methods").
† Numbers in parentheses denote range.

TABLE 6.—Response to autologous blast cells in MLC

Patient No.	Therapy	Response before therapy	Weeks after therapy	Stimulation ratio after therapy	Remission (days)
1	MTX	1	1	1	63
2	BCG	NT*	8	2.8	62
3	BCG	0.3†	3	2.8	91
4	MTX	1	4	4	151
5	BCG	NT	4.5	8‡	204
6	MTX	1.5	1	5	
			12	7.5	305

* Not taken.
† Technically unsatisfactory, since cells did not stimulate either patient or unrelated donor.
‡ Blast cells obtained from cerebrospinal fluid at the time of meningeal relapse.

TABLE 7.—Mean immunoglobulin levels (mg/ml)

	IgG	IgA	IgM
MTX			
Before therapy	10.2	2.2	1.14
After therapy	12.2	2.6	0.99
BCG			
Before therapy	8.0	3.5	0.93
After therapy	8.0	2.0	0.90
Normal			
Mean ± SE	8.9±0.7	2.0±0.3	1.68±0.3
Range	4.3–13.9	0.8–4.7	0.5–4.0

Cytotoxic Antibodies to HL-A

Two patients on the MTX arm of the protocol had antibody against a number of HL-A specificities before immunization was begun. In 1 who was positive against 25% of cells tested, no follow-up serum was obtained. In the other, whose serum was initially cytotoxic to 98% of test cells, a broad reactivity persisted throughout the treatment period so that at the end of the period cytotoxic activity was still present to 94% of the test cells. One patient on the BCG arm of the protocol, whose serum was initially nonreactive, developed positive reactivity to 44% of cells in a test panel with specificity against either HL-A 7, 10, or 11. The cells used to immunize her were HL-A 2, 10, and 7. This patient was one of those whose skin reaction converted from negative (0 mm) to positive (5 mm) 7 weeks after immunization. She also showed a dramatic increase in blastogenic response, from a ratio of 8 before immunization to 84 after 4 months with stimulation by immunizing cells. She had a 6½-month remission. It is likely that specific immunization to HL-A antigen did occur as the result of immunization in this patient.

DISCUSSION

No difference in therapeutic effect was seen between the MTX and BCG regimens. In a previous protocol with similar patients, the median remission duration was about 60 days (*11*). Patients with relatively far advanced disease are obviously not ideal candidates for "curative" immunotherapy. Their tumor load, even at the time of bone marrow remission, is presumably relatively large, and it is estimated that immunotherapy in animal systems can eradicate only small quantities of cells. Indeed, Mathé (*1*) observed a lack of clinical response to immunotherapy in late-stage patients given a regimen which was apparently successful in prolonging remission of acute leukemia in patients with the first visible phase of the disease.

There was relatively little toxicity from the immunotherapy. Occasional malaise was the only side effect from cell injection. Only 1 patient developed serologic evidence of specific sensitization to HL-A antigen on the immunizing cells. The BCG-treated patients had local pain during scarification; but the only systemic abnormalty was an elevation in alkaline phosphatase levels which appeared while the patients were receiving asparaginase and did not revert to normal during BCG therapy. There was evidence of some cellular liver damage in 4 patients on MTX, who developed abnormalties of serum transaminases during this maintenance phase.

Tumor growth was not enhanced with either regimen.

There was a general increase in skin test reactivity in both groups of patients. Initial skin tests were performed during remission, i.e., 5–7 days after asparaginase therapy was discontinued. Therefore, we do not think that the increase in mean diameter of skin test reactions and the number of individuals who converted from negative to positive reflects merely the immunosuppressive effect of asparaginase on the initial skin tests. Increase in skin test reactivity was seen in other patients receiving BCG (*12, 13*) and in patients given a comparable number of skin tests but no BCG (*14*).

The in vivo response to immunizing cells increased to the same degree as the response to other recall antigens. Those patients who were or became reactive to immunizing cells had longer remissions than those who were or became nonreactive to them. It is not clear whether the immunotherapy produced reactivity to immunizing cells and therefore produced a longer remission, or whether a longer remission allowed skin test reactivity to express itself. Although too few patients were tested against their own cell membranes to make a meaningful correlation with remission duration here, it is clear that positive skin test reactivity to leukemia cell membranes is associated with a better prognosis for the duration of that particular remission than nonreactivity. This correlation of remission time with positive skin test reactivity against leukemia blast cell membranes has been seen previously for autologous cell membranes (*4*).

The in vitro immune response in the MTX-treated group shows a pattern which has been described by several authors. Cheema and Hersh (*15*) originally described a rebound in immune response to standard recall antigens with intermittent chemotherapy. Harris and Stewart (*16*) observed this effect in response to tissue antigens in MLC; Halterman and Leventhal (*17*) and Leventhal et al. (*2*) noted the same type of pattern in response to autologous tumor antigens. This transient increase in immune response to antigen in vitro after chemotherapy means that many chemotherapeutic regimens which may be sufficiently cytotoxic to have antitumor effect need not be considered immunosuppressive in the classical sense. The fact that the adjuvant effect seen in both groups of patients in this study was equivalent may mean that immunochemotherapy, i.e., antitumor immune response, stimulated either with drugs alone or with a combination of drugs and other immune adjuvants, is probably the most logical way of treating many tumors not responding to either regimen alone. This lack of immunosuppression with properly scheduled cytotoxic drugs is important in view of the animal models for immunotherapy which show that an individual animal incapable of demonstrating an immune response to a classical adjuvant such as BCG is not able to mount a therapeutic antitumor response when that adjuvant is administered with the tumor (*18, 19*).

In the in vitro assays, specific reaction to any stimulus, i.e., to immunizing or autologous cells, was increased to the same degree as the nonspecific response to recall antigens. This may indicate a general adjuvant effect in both groups of patients. The correlation between degree of in vitro reactivity and remission duration is suggested; again, however, the number of patients is small, and further studies are required before one can say that the in vitro blastogenic response measures a reaction that has an in vivo clinical correlate. In a previous study (*2*), we did not find a correlation between positive blastogenic responses and remission duration; however, the patients in that study were not followed serially.

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BCG Vaccination and Leukemia Mortality[1]

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SUMMARY—In Chicago from 1964 through 1969, one death from leukemia was recorded among 54,414 black infants 0–6 years old vaccinated at birth with BCG: 0.31/100,000/year. In contrast, 21 deaths were reported among 172,986 black infants of a similar age, who were not vaccinated: 2.02/100,000/year. The difference was statistically significant ($P = 0.04$). This study is retrospective so that no firm conclusions can be drawn, but it suggests that BCG be considered a vaccine against acute leukemia.—Natl Cancer Inst Monogr 39: 189–192, 1973.

DESPITE GREAT strides made in radiation and chemotherapy of cancer and leukemia in the last 25 years, C. Gordon Zubrod, Scientific Director for Chemotherapy of the National Cancer Institute in Bethesda, stated at the National Conference on Cancer and Chemotherapy (*1*) that prolongation of life (> 5 years' expectancy) has been possible in only 10 types of malignancies; the remaining patients have been made more comfortable but their life expectancy has not been increased (average 3 yr). It is understandable, therefore, why Frank Rauscher, Jr., Director of the National Cancer Institute, stated (*2*) that "Prevention is clearly the number one priority in all of cancer research."

From recent experimental and clinical studies, one may reasonably conclude that immunologic mechanisms play a role in host resistance to neoplasms. Hypothetically, hypersensitivity and homograft rejection may be the primary mechanisms for natural defense against neoplasia (*3, 4*). There is a constant surveillance against natural somatic mutation, probably on an immunologic basis. This mechanism may account for the suppression of incipient neoplastic cell foci and thus prevent clinical manifestations of cancer.

Robert A. Good, Director of the Sloan-Kettering Research Institute in New York City, stated (*5*) that "Cancer or potential cancer probably arises in everyone of us every day of our lives in the form of mutant cells. But they are promptly and effectively eliminated by lymphoid cells which recognize the foreignness of the mutants and act accordingly. When the policing cells are defective, however, such mutants may gain a foothold."

Spontaneous regressions of carcinoma of the breast, prostate, kidneys, and lungs have been

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[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

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observed (*6*). Remissions from carcinoma of the breast, prostate, or lungs after surgery, radiation, and chemotherapy may last from 5 to 25 years followed by recurrence. It is postulated that as long as the immunologic mechanism of the body functions properly, and is not overtaxed, tumor proliferation is suppressed. Furthermore, a higher incidence of lymphomas and other neoplasms occurs in persons with immunologic deficiencies, such as acquired hypogammaglobulinemia after exposure to immunosuppressive drugs (*7*).

Studies from this and other laboratories have shown that BCG is a potent stimulator of the host's immunologic mechanism through the lympho-reticuloendothelial system (lympho-RES) (*8–12*). Clinically, BCG has proved to be highly effective against tuberculosis (*13–15*) and leprosy (*16*). More recently BCG has been somewhat successfully applied in the treatment of acute leukemia in children (*17, 18*), a variety of cancers in adults (*19*), Hodgkin's disease (*20*), and melanocarcinoma (*21*). Experimentally, BCG is effective when given before tumors or leukemia is transplanted in the host (*9–11*)

In a previous publication (*22*) from this laboratory, evidence was presented not only that BCG is effective in the treatment of cancer and leukemia when in remission but also that it can be considered a vaccine against neoplasia, because BCG stimulates the lympho-RES and thus increases the immune surveillance.

The present study compares the mortality rates from leukemia (all forms) in Chicago among infants 0–6 years old, vaccinated at birth with BCG, with rates in a similar nonvaccinated population. The period surveyed was 1964 through 1969 (the only available computerized data).

MATERIALS AND METHODS

Newborns in the maternity division at the Cook County Hospital were vaccinated with BCG when 2 or 3 days old. The multiple puncture method was used. The concentration of the vaccine was 2×10^8 organisms in 1-ml vials (*23*). This was a service program for vaccination against tuberculosis. The number vaccinated largely depended on the personnel available. Thus, about 50–75% of the infants born at the hospital were vaccinated. A certain percentage of the inoculated infants returned to the clinic at regular intervals for follow-up. There, the reactions to the vaccine, the results of tuberculin testing, and the general health of the children were determined.

The leukemia mortality (all forms) was obtained through the Department of Registration and Statistical Services of the Chicago Board of Health. Only the black population was considered in this analysis, since 97% of the infants born at the hospital are black. The leukemia deaths were checked against the BCG vaccination records. The nonvaccinated population was culled from the United States census tables for Chicago. The number at the various age groups was estimated from the 1970 census, less the number vaccinated.

RESULTS

There were 21 deaths from leukemia from 1964 through 1969 in 172,986 black, nonvaccinated infants, 0–6 years old: 2.02/100,000/year. In contrast, there was 1 death from leukemia in 54,414 black infants of the same age vaccinated at birth: 0.31/100,000/year. This difference was statistically significant ($P = 0.04$); P was calculated as the probability of the exact treatment of a fourfold table (*24*).

In table 1, the results are given according to age: $<$ 1 year old, 1–3 years old, and 4–6 years old. This division corresponds to the step-like increase in mortality from leukemia. The 1 vaccinated infant who died of acute lymphatic leukemia was vaccinated April 1, 1963, and died on December 14, 1968. This child was not seen in the clinic so that its reaction to tuberculin was not known. A positive tuberculin reaction is considered as presumptive evidence that the vaccination is still operative. In the assay group of children who return to the clinic at regular intervals, 96% of the vaccinated children react to 5 TU of tuberculin 3–6 months after vaccination. Six years after vaccination, 76% react. A test was considered positive by the Mantoux method when the induration was ≥ 6 mm in diameter, and by the tine test when $\geq$ papules were ≥ 2 mm in diameter.

DISCUSSION

A similar study was reported from the Institute of Microbiology of the University of Montreal (*25*). It was found that death from leukemia was half as common among the BCG-vaccinated as among the nonvaccinated individ-

TABLE 1.—BCG vaccination and leukemia mortality rates, Chicago, 1964–69

Age (yr)	Nonvaccinated			Vaccinated		
	Population*	Deaths	Rate/ 100,000/ year	Population	Deaths	Rate/ 100,000/ year
<1	21,901	0	—	7,820	0	—
1–3	68,824	8	1.94	26,482	0	—
4–6	82,261	13	2.64	20,112	1	0.83
Total	172,986	21	2.02	54,414	1	0.31

* All black population.

uals ($P < 0.001$) <15 years of age for each of the years 1960–63. All data were derived from Quebec, Canada. This work was criticized because all children were not vaccinated at birth, and thus the vaccinated children were not in that group throughout their lives as was the control group. Therefore, a second analysis was done in children 0–4 years old, 90% of whom were vaccinated at birth. In this group, the death rate from leukemia was less than one-half in the vaccinated as in the nonvaccinated, similar to the overall statistics (*26*). Other criticisms made (*27*) on the population basis of the study do not apply to the present study because of the restricted population used.

In the present study, all infants were vaccinated at birth and the vaccinated and the nonvaccinated were black, from the same areas of Chicago. Thus the 2 groups were more or less homogeneous. However, it is not known how many vaccinated subjects moved from Chicago or how many nonvaccinated subjects were born elsewhere. Of 11,989 vaccinated infants that were followed intensively (clinic, etc.), 169 (1.4%) moved out of town and were lost to follow-up. It is unlikely that this would be a serious flaw in the statistics.

In retrospective studies of large BCG-vaccinated populations, results must be carefully evaluated. The ideal studies will be those in which children are vaccinated at birth and followed from infancy. Depending on the locality, the rate of infection by atypical or typical mycobacteria will vary. In a Chicago study, for example, it was found that, whereas newborns did not react to the typical or atypical tuberculins, they became positive as early as 2–5 years of age (4% to purified protein derivative-ammonium sulfate precipitate-Battey and 22% to purified protein derivative-Gause) (*28*). Such infection with mycobacteria may also influence the resistance to leukemia.

Those vaccinated and reinfected by tubercle bacilli experience a stimulation of the lympho-RES by specific antigen; however, it does not follow that they will retain their nonspecific resistance against neoplasia. Experiments have shown that large deposits of specific antigen are necessary to maintain nonspecific immunity (*12*), and BCG has not been cultured from the organs of vaccinated hosts > 1 or 1½ years after vaccination (*23*). Thus, if BCG vaccination is to be considered against neoplasia, relatively frequent revaccination will be necessary, possibly at yearly intervals.

Retrospective studies at best have many pitfalls. Statistically oriented, controlled studies must be carried out before definite conclusions can be drawn. To expedite future controlled studies, one may consider vaccinating newborns, families in which there is a history of cancer, and older groups (≥ 40 yr old), where the immune system is believed to become less and less effective (*5*).

The vaccination methods recommended are multiple puncture (*23*), one of its modifications, the scarification method, or aerosol vaccination (*29*), an effective method of stimulating the lympho-RES and producing an increased resistance against specific infection (*30*).

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DISCUSSION

P. Alexander: I have 2 questions for Dr. Weiner. 1) In your report of the immunotherapy trial on patients with acute lymphoid leukemia, you stated that all these children had received as immunotherapy leukemia cells and BCG. Could you clarify this statement, since Dr. Mathé in his report [1] on this trial stated that the 20 patients given immunotherapy were divided into 3 groups, one of whom had only BCG, one of whom had only allogeneic leukemia cells, and one of whom had both allogeneic leukemia cells and BCG? In this paper he stated that there was no significant difference in the response of the patients in these 3 groups. At some stage, I believe all the patients were placed on a uniform immunotherapy protocol; but it is not clear when this occurred, nor is it clear what initial—and possibly the most important—

[1] Mathé G: Approaches to the immunological treatment of cancer in man. Br Med J 4:7–10, 1969.

treatment the long-term survivors (on whom you reported in your paper) were given.

2) My second question is: How do you derive 5-year survival data figures from a study in which patients were still being entered in 1971?

R. Weiner: I can answer your second question but not your first.

These are actuarial curves which show the patient's length of remission or survival. With this method of reporting results, the patients who enter at any time during the study are effectively moved back to the origin of the curve. The further to the right, the longer their duration in the study population. The survival or remission at 8 years represents only those patients whose treatment was begun 8 years ago.

D. W. Weiss: Dr. Rosenthal, I was interested in your statistics; but if I am not badly mistaken, there have been other attempts, including one or two by the Medical Research Council of Great Britain, to look for correlations between BCG immunization and leukemia and other neoplastic illnesses. A depressed cancer incidence was not found in vaccinated individuals in some of these studies. How does one account for the difference in these observations?

S. R. Rosenthal: As I mentioned in the paper, it is very important to know what group one is comparing. In our nursing students, for example, were vaccinated and nonvaccinated groups. When the nonvaccinated students left training, they were practically all tuberculin positive. They didn't develop tumors until 10 years later. By that time, all the effect of the vaccine was probably dissipated. The tumor incidences did not differ among the BCG-vaccinated and nonvaccinated groups of nurses.

In children, atypical tuberculin reactors are found all over the world. In some areas, up to 70, 80, or 90% react. These atypical mycobacteria may affect neoplasia. In our study, infants at 2 years of age began to react to atypical tuberculins. Thus acceptable studies must begin at birth and must be followed.

A study should be done—as I mentioned in the paper—with older people when there is a waning of the effectiveness of the reticuloendothelial system and also in those having a genetic history of cancer. D. Morton and F. C. Sparks, for example, using as antigen a lipopolysaccharide from an osteosarcoma, found that 92% of the patients reacted to this antigen. Also, 55% of those in the immediate family gave a positive test, but only 8% in the general population. Thus, there appears to be a herd-like infection.

L. Chedid: Dr. Leventhal, do you have any idea on the comparative responsiveness of children treated with tuberculin in the United States and in France? This could answer both questions that have been raised depending on whether the population considered had been previously sensitized or not sensitized to BCG.

B. G. Leventhal: All of our patients were tuberculin negative at the beginning of the study, which is generally true for our patient population. Even the majority of the adults were tuberculin negative at the time of testing; however, they almost always were reactive to some skin test antigens.

Chedid: In France, children are responsive to tuberculin, and immunization with BCG is compulsory.

D. B. Windhorst: Dr. Rosenthal, in more than 12 years in the medical community of Chicago, it is my general impression that the socioeconomic status of children born at Cook County Hospital is distinctly different from that of black children born in other hospitals in the city. Have you measured this parameter in regard to the incidence of leukemia? I am happy to know that children born in Cook County have one advantage, at least.

Rosenthal: Most of our so-called nonvaccinated also came from the county hospital because we don't have enough personnel to vaccinate everybody.

I. Djerassi: It's very interesting, that the patient population was black in whom the incidence of acute leukemia is biologically lower than in the white patient. Could this have any impact?

Rosenthal: We have also compared the white and the black population. When we considered the blacks and the whites together, the mortality rate difference between the vaccinated and nonvaccinated groups was 9 times higher in the nonvaccinated. In other words, the rate was higher in the whites.

E. Klein: So it was adjusted?

Rosenthal: Yes.

W. D. Terry: I believe Dr. Rosenthal is making his data available to M. Schneiderman and his colleagues here at the National Cancer Institute for statistical analysis. What is really required is that epidemiologists and statisticians should sit down and spend many months of hard work to qualify these data.

B. Zbar: Dr. Leventhal, in Dr. Mathé's original study, he compared unmaintained remission with BCG with unmaintained remission without BCG. In the study you did, is it possible on the basis of your experience to know that BCG was better or worse than nothing?

Leventhal: In a comparable previous group of patients, not run simultaneously, the median remission in these patients on such a protocol was 60 days. So the 90-day average looks perhaps a little better than nothing; it is certainly not worse than nothing. The point to be gathered from my paper is that there was an adjuvant effect probably from both BCG and intermittent methotrexate.

J. A. Peters: I would like to comment on Dr. Rosenthal's agreement that the incidence of leukemia in blacks is lower and also that it does not peak at the age group that it does in whites. One possibility is a flaw in the records, and this could be ascertained by testing the hypothesis that BCG may protect against deaths from automobile accidents or some such unrelated causes of death.

Unidentified speaker: Dr. Leventhal, you stated that a positive response to tumor cells in the blastogenesis system would be beneficial. Do you have any clinical evidence for that? Since blastogenesis could occur just as well by, say, a B-lymphocyte that was going to make a blocking antibody, what is your evidence that this could be beneficial, and why are you sorry you didn't see that?

Leventhal: I don't have any clinical evidence, and I

agree with you that blastogenesis in a lymphocyte culture is a recognition phenomenon, and not the type of destructive phenomenon that we'd like to be assessing in these patients.

Djerassi: In all fairness to Dr. Mathé, we cannot necessarily draw conclusions in comparison to your particular study. As a matter of fact, the 2 results are complementary and supplementary, in a way. His patients differed quite radically from the type of patients you have treated.

Leventhal: I stated that right at the outset.

Djerassi: Yes, but I am trying to say the results are supplementary in that you did demonstrate that a small tumor is needed to elicit an immunologic response.

Leventhal: A tumor response but not an immunologic response.

M. L. Murphy: Dr. Leventhal, how many blacks were in the BCG group?

Leventhal: None.

Murphy: We saw marked keloid formation in one black who had eczematous inflammation.

M. Tishler: Dr. Weiner, is the Hiu fraction 1 in animal tumors similar to BCG?

R. Weiner: Neither Hiu fraction 1 nor fraction 2 has antitumor activity.

L. Nathanson: I may have missed the number of allogeneic cells you used in your vaccine.

Leventhal: Forty million, from a single donor.

Immunotherapy of Chronic Myelocytic Leukemia[1, 2]

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SUMMARY—Thirty-two patients with chronic myelocytic leukemia were given $\geq$ 3 vaccinations with mixtures of BCG and cultured cells of lines from leukemic patients in a blastic state. In 15 evaluable patients with uncomplicated Philadelphia chromosome-positive leukemia under good control, survival was unequivocally prolonged ($P < 0.01$). In trials on 11 poor-risk patients (principally, patients first immunized after myeloblastic transformation was observed), immunotherapy was probably or definitely beneficial in 5 patients.—Natl Cancer Inst Monogr 39: 195–198, 1973.

IN 1964, one of us (JTG) established a cultured cell line from peripheral blood of a patient with acute myeloblastic leukemia (AML) (*1*). The cells were believed to be myeloblasts and appeared to have some antigen in common with buffy coat of patients with chronic leukemia. Because of this, we attempted to prevent or delay death from blastic crisis by immunizing patients with these cultured cells. First we injected irradiated cells subcutaneously or intramuscularly. This technique caused production of antibodies to the target cells but not delayed hypersensitivity. Since our goal was to induce a cellular immune response, this technique was abandoned. In 1965, we adopted our present immunization technique based on studies we did on transplanted tumors of rats. The target cells are mixed with living BCG organisms as adjuvant and are injected intradermally. Initial vaccinations are given 3–6 weeks apart. The interval between vaccinations is extended gradually, and maintenance immunotherapy is usually 3 vaccinations a year.

We now use 2 cell lines for immunization. The RPMI 6410 line (*1*), established from blood of a patient with AML, was used during the first 5 years of this study. This line had a normal diploid karyotype and was subsequently shown to consist of lymphoid cells. The RPMB 7642 line, used since 1970, was established from peripheral blood of a patient in blastic crisis of chronic myelocytic leukemia (CML). The RPMB 7642 cells were originally myeloblasts; they were Philadelphia chromosome (Ph')-positive and immunoglobulin-negative. Within 3 months, these cells were replaced by Ph'-negative, immunoglobulin-positive cells. We believe this represents overgrowth of the original cells by concomitantly established lymphocytes, rather than transformation of myeloid cells into lymphoid cells.

A total of 32 patients have received $\geq$ 3 vacci-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

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[3] Deceased.

nations to date (table 1). Seventeen of them had uncomplicated cases of well-controlled Ph'-positive leukemia. Fifteen were classified as poor risks. The latter included 8 Ph'-positive patients first immunized after myeloblastic transformation had already occurred, 4 classified as poor risks for other reasons, and 3 Ph'-negative patients. One of the Ph'-negative patients had subacute leukemia. All patients have continued to receive antileukemic chemotherapy as indicated by their hematologic conditions.

TABLE 1.—Patients given ≥3 vaccinations

A. Uncomplicated Ph'-positive leukemia, in good control at the time of initial vaccination—17 patients

B. Poor-risk patients—15 patients
   1. First immunized *after* myeloblastic transformation —9 patients (8 Ph' positive, 1 Ph' negative)
      (a) Blastic state substantially reversed before initial vaccination—6 patients
      (b) Blastic state persisted, immunized after reduction of white blood cell count—3 patients
   2. Other Ph' positive—4 patients
      Myelofibrosis (2); busulfan resistant (1); marked basophilia (1); leukemic tumor masses (1); hyperdiploid karyotype (1)
   3. Other Ph' negative—2 patients

Table 2 summarizes the changes in delayed-hypersensitivity responses of these patients. There are 2 categories: 1) responses to antigens of the vaccine mixtures [purified protein derivative (PPD), target cell homogenates, fetal calf serum (FCS)] and 2) responses to antigens unrelated to any component of these vaccines. Increases in both types of skin test reactions were recorded; they correlated with the number of vaccinations received. Table 3 illustrates this progressive increase in delayed skin test responses in 19 patients given ≥ 6 vaccinations. Although none of these patients was anergic before immunization, three-fourths of all prevaccination skin test responses were negative or weak (+). After 3 vaccinations, the distribution of skin test responses had changed markedly, with a sharp mode at moderate (++) response. After ≥ 6 vaccinations, the mode shifted to strong (+++) response, and a significant number of very strong (++++) responses were recorded.

We screened patients for evidence of comparable stimulation of antibody production. Serum

TABLE 2.—Increases in delayed-hypersensitivity responses in 28 immunized patients

Antigen	Number of patients with increase in delayed skin test reaction/No. patients tested	
	After 3 vaccinations	After 6+ vaccinations
Related to vaccines:		
Tuberculin PPD	22/24	14/16
Target cell homogenates	13/17	11/13
FCS	8/15	11/12
Unrelated to vaccines:		
Mumps	8/23	10/14
Varidase	7/16	7/9
Monilia	5/18	8/11
Trichophyton	4/18	4/10
One or more of above	13/24	12/15

TABLE 3.—Serial determinations of delayed skin test responses to 7 antigens in 19 patients given ≥6 vaccinations

Skin test response*	Percent of skin test responses		
	Before immunization (93 skin tests)	After 3 vaccinations (92 skin tests)	Latest tests (120 skin tests)
Negative	42	17	16
Weak (+)	32	19	13
Moderate (++)	18	39	27
Strong (+++)	6	18	29
Very strong (++++)	2	7	15

* Classification of skin test responses: +—tuberculin—positive to second-strength PPD but negative to intermediate PPD; other antigens—erythema ≥1.0 cm without induration, or induration of 0.5–0.9 cm. ++—tuberculin—positive to intermediate PPD, 0.5–2.0 cm of induration; other antigens—induration of 1.0–2.0 cm. +++—tuberculin—positive to first-strength PPD, or >2.0 cm of induration to intermediate PPD; other antigens—induration of 2.1–4.0 cm. ++++—tuberculin—>1.5 cm of induration to first-strength PPD; other antigens—>4.0 cm of induration.

electrophoretic patterns did not change significantly. Table 4 gives some data for our most intensively immunized patients, who received up to 22 vaccinations. Mean IgA values were somewhat below average normal levels for this method; IgG and IgM values were normal. We know that patients given cultured cells intravenously develop easily demonstrable precipitating antibodies to FCS (*2*). Our patients showed no such precipitins by immunoelectrophoresis or conventional Ouchterlony assays. With a more sensitive technique (*3*), however, we could detect traces of antibody in most patients. We have no

TABLE 4.—Immunoglobulin and antibody determinations for patients immunized repeatedly

Mean immunoglobulin values (most recent determinations for 14 patients given 6–22 vaccinations):		
IgG	1112 mg/100 ml	(normal, 1200±300 mg/100 ml)
IgA	181 mg/100 ml	(normal, 288±121 mg/100 ml)
IgM	75 mg/100 ml	(normal, 80± 29 mg/100 ml)
Antibodies to FCS:		
By immunoelectrophoresis		0/11 patients
By conventional Ouchterlony technique		0/11 patients
By concentration-immunodiffusion technique (*3*)		5/7 patients

measurements of antibody against our target cells, but the data for FCS suggest that such antibodies would be present in only low titer.

The data in tables 2 through 4 indicate that our goal—stimulation of delayed hypersensitivity to target cell antigens, with little antibody production—was achieved. Also, these patients demonstrated a substantial and persistent general increase in cellular immune reactivity.

The therapeutic effects of these immunizations were evaluated separately for the poor-risk patients and the patients with uncomplicated leukemia. In the former, the major clinical category contained 9 patients (8 Ph′-positive and 1 Ph′-negative) who were first immunized after myeloblastic transformation had occurred. Table 5 summarizes the results in this group. In 3 patients, myeloblastic transformation could not be reversed by combination chemotherapy and splenectomy, and vaccinations were started during an overt blastic state, after the total white blood cell count was reduced to normal or leukopenic levels. There was no evidence that immunotherapy was beneficial in these patients. It is too early to evaluate 2 patients in whom immunization was started after the blastic state was substantially reversed; 1 patient (#15) experienced no clinical benefit. In the other 3 patients, however, the addition of immunotherapy produced substantially longer remissions than we have ever achieved with chemotherapy and splenectomy alone. Two of these patients, #3 and 14, died of recurrent myeloblastic transformation, 40 and 18 months, respectively, after initial vaccination; the third patient, #16, is alive and has excellent remission from maintenance chemotherapy, 24 months after his first immunization.

Of the 6 patients classified as poor risks for other reasons, immunotherapy clearly failed to prolong survival in 2 patients, was deemed probably beneficial in 2, and cannot yet be evaluated in 2.

TABLE 5.—Fate of patients first immunized after myeloblastic transformation had occurred

Patient No.	Current status (months after first vaccination)
	A. Blastic state unresponsive to combination chemotherapy, splenectomy
24 (M.T.)	Dead (5 months)
26 (L.M.)	Dead (2 months)
32 (L.E.)	Alive; poor hematologic conditions (2 months)
	B. Blastic state substantially reversed before immunization
3 (J.C.)	Dead; recurrent blastic state (40 months)
14 (P.S.)	Dead; recurrent blastic state (18 months)
15 (A.L.)	Dead; recurrent blastic state (6 months)
16 (A.T.)	Alive; excellent remission, on maintenance chemotherapy (24 months)
28 (A.F.)	Alive; partial remission (5 months)
31 (H.S.)	Alive; partial remission (2 months)

The patients with uncomplicated Ph′-positive leukemia were a homogeneous group to which conventional statistical analysis could be applied. These 17 patients entered the immunotherapy program at varying times after diagnosis (mean, 1.8 yr). To simplify calculations of survival, they were assigned a hypothetical date of diagnosis of 1 year before initial vaccination. Their survival was compared with that of unimmunized Ph′-positive patients, treated at the Roswell Park Memorial Institute, whose leukemia was diagnosed between 1960 and 1969, and who were doing well 1 year after diagnosis. The results are shown in table 6. The survival advantage for the immunized patients was obvious and highly significant ($P < 0.01$).

The survival pattern of our unimmunized patients was virtually identical with that of a busulfan-treated series of patients recently reported from Great Britain (*4*), if the British first-year deaths are excluded. Survival in both

TABLE 6.—Survival of immunized and unimmunized patients with uncomplicated Ph'-positive leukemia

Years after diagnosis*	Immunized patients			Unimmunized patients†		
	Number		Percent alive‡	Number		Percent alive‡
	At risk	Alive		At risk	Alive	
0	17*	17	100	28†	28	100
1.0	17*	17	100	28†	28	100
1.5	15	15	100	28	28	100
2.0	15	15	100	28	26	93
2.5	14	14	100	28	21	75
3.0	12	12	100	27	18	68
3.5	9	8	90	24	10	47
4.0	9	8	90	24	9	42
4.5	7	5	77	23	7	37
5.0	6	4	61	22	3	20
5.5	6	4	61	22	2	13
6.0	6	4	61	22	2	13
6.5	6	4	61	20	2	13
7.0	6	4	61	20	2	13

* For immunized patients, date of diagnosis was assumed to be 1 year before initial vaccination.
† Patients not in good hematologic control 1 year after diagnosis were excluded.
‡ Calculated by the life table method.

these series was somewhat better than that usually reported in patients with GML (*5*). Thus the differences in table 6 did not reflect unfavorable statistics in our control group but, rather, a significant and substantial prolongation of life in the immunized patients.

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BCG Immunotherapy in Burkitt's Lymphoma: Preliminary Results of a Randomized Clinical Trial[1, 2, 3]

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SUMMARY—Forty-two previously untreated patients with Burkitt's lymphoma were treated with 2 doses of intravenous cyclophosphamide, 40 mg/kg, 2 weeks apart. Then 24 complete responders were randomized to no further treatment (11 patients) or to 10 weeks of BCG immunotherapy (13 patients). Both groups were comparable in age, sex, and clinical stage. Six patients (46%) in the BCG group and 7 controls (64%) had relapses. Of the patients having relapses, 4 in the BCG group and 1 control had neurologic involvement as the only manifestation of disease. Delayed-hypersensitivity response to a variety of recall skin test antigens was significantly potentiated in the BCG group as compared to controls. The preliminary results of this study reveal no protective or harmful effect of BCG immunotherapy on the relapse frequency or remission duration in Burkitt's lymphoma, although there is a suggestion that BCG prevents extradural relapse.—
Natl Cancer Inst Monogr 39: 199–202, 1973.

BURKITT'S LYMPHOMA is one of the few tumors of man that exhibits complete and durable remissions with chemotherapy as the sole treatment modality (*1*). A recent analysis of the natural history of the disease following chemotherapy reveals that: 1) Complete remissions can be achieved in about 90% of patients; 2) approximately two-thirds of these patients will have relapses; and 3) defective nonspecific and tumor-specific immunity is associated with relapse (*2*). In an effort to prevent relapse through immunologic means, patients with Burkitt's lymphoma were treated to remission with cyclophosphamide and then randomized to no further treatment or to 10 weeks of immunotherapy with BCG vaccine. The present report summarizes preliminary results of this ongoing clinical trial.

PATIENTS AND METHODS

All patients with previously untreated Burkitt's lym-

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[3] We thank Mrs. P. Magrath for technical assistance, Mr. G. Angala for assistance in patient follow-up, and Dr. A. Z. Bluming for participation in the initial study design.

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phoma diagnosed histologically and cytologically were candidates for study. Clinical staging and evaluation were outlined in (*2, 3*). Briefly, Stage I–II refers to localized tumor(s), Stage III to visceral (generalized) tumor deposits, and Stage IV to neurologic involvement manifested as malignant cells in the cerebrospinal fluid.

After diagnosis and clinical evaluation, all patients received intravenous cyclophosphamide, 40 mg/kg, which was repeated in 10–14 days, following recovery of myelotoxicity. Two weeks after the second dose of cyclophosphamide, patients with complete remissions were randomized by stage to no further treatment or to 10 weeks of BCG immunotherapy. The latter procedure was administered exactly according to the scarification method of Mathé, using vaccine obtained from the Pasteur Institute, Paris (*4*). BCG was administered every 4 days for the first 7 doses and weekly thereafter for a total of 10 weeks.

Skin tests using common recall antigens, an autologous tumor extract, and control lymphocyte extract were performed serially on patients at admission, at the time of BCG randomization (just prior to BCG administration), midway through the BCG course, and after immunotherapy. Control patients were tested at comparable intervals. Skin test methodology and reagents were described in (*5, 6*).

RESULTS

The study was initiated in February 1971, and all evaluable patients (table 1) admitted up to June 1972 were analyzed in the present report. This allows a minimum interval of 3 months of follow-up. Of 42 patients admitted during this period, 18 were not evaluable (table 1).

TABLE 1.—BCG immunotherapy in Burkitt's lymphoma

Patient evaluation	Number	
Admitted (2/71–6/72)	42	
Not evaluable	18	
Died <1 week		3
Partial response		10
Early relapse		4
Default		1
Evaluable	24	

Of 24 evaluable patients, 13 were randomized to BCG immunotherapy and 11 served as untreated controls. Both groups were comparable in age, sex, and clinical stage (table 2).

Relapse occurred in 6 of 13 (46%) patients in the BCG group and in 7 of 11 (64%) controls (table 3). When patients having relapses were analyzed by tumor site, neurologic involvement, manifested as malignant cells in the cerebrospinal fluid with or without cranial neuropathy, was the only evidence of recurrent tumor in 4 of 6 patients in the BCG group, as compared to 1 of 7 controls. The overall frequency of central nervous system involvement in both groups was similar. These differences in relapse frequency or site were not statistically significant (Fisher's exact probability test).

TABLE 2.—Comparison of BCG patients and controls

	BCG	Control
Number	13	11
Median age (yr)	7	6
Male/female	7/6	6/5
Stage:		
I, II	3	2
III	9	8
IV	1	1

TABLE 3.—Relapse frequency in BCG group and controls

	Number	Total No. of relapses (%)	Number of relapses excluding central nervous system (%)
BCG	13	6 (46)	2 (15)
Control	11	7 (64)	6 (55)

The remission duration in both groups was comparable. Patients in the BCG group had relapses at 8, 9, 9, 9, 18, and 30 weeks and controls at 5, 9, 13, 14, 30, 33, and 36 weeks from initial treatment. Only 2 patients in the entire series have remained in sustained remission $>$ 1 year from initial treatment, and all others still in remission are at a high risk of having a relapse. Three patients have died after having relapses, 2 from the BCG group and 1 from the control group.

The results of serial skin tests are summarized in table 4. All BCG-treated patients demonstrated conversion of the tuberculin test. Excluding tuberculin conversion, there was a net increase of 30% positive skin tests (induration $>$ 5 mm diameter at 48 hr) to other recall antigens during the period of BCG immunotherapy. This was significantly different from results in

the control group who showed −9% net conversion (i.e., conversion from positive to negative) on serial testing (table 4). The effect of BCG on autologous tumor extract cannot be assessed yet due to the few patients in the BCG group in whom adequate tumor was available on admission.

TABLE 4.—Serial skin tests with recall antigens

	Number of patients	Number of positive tests		Percent change
		Pre-BCG	Post-BCG	
BCG	9	8/27	16/27	+30
Control	7	11/21	9/21	−9

No patient developed any obvious toxic effect during or after BCG immunotherapy, aside from discomfort during the scarification procedure. The BCG scars evolved through stages of mild induration with some pruritus, followed by regression, slight residual hyperpigmentation, and ultimately total disappearance within 2–3 months. No scars became secondarily infected, and no patient developed a systematic illness resembling tuberculosis.

DISCUSSION

The rationale for the use of BCG in this trial was the well-documented stimulatory effect of this procedure on the primary and secondary delayed-hypersensitivity response (*7*). Since relapse in Burkitt's lymphoma is associated with "nonspecific" as well as tumor-specific anergy (*2, 5, 6*), potentiation of the immune response by BCG during the high-risk period following remission induction might prevent relapse. Stimulation of tumor-specific cytotoxic mechanisms may assist the identification and destruction of residual tumor cells during this initial period of low tumor cell burden and absence of immunosuppressive chemotherapy.

While the results of this study confirm the "nonspecific" stimulatory effect of repeated BCG vaccination on delayed hypersensitivity, this treatment apparently did not affect the frequency of relapse. However, a substantially greater proportion of patients in the BCG group had neurologic manifestations as the only evidence of relapse. The control nervous system is a notorious immunologic sanctuary, and these patients might not be considered treatment failures from an immunotherapeutic point of view. One may speculate that these patients were destined to have relapses in a manner similar to controls, but the reappearance of "systemic tumor" was effectively prevented by BCG, while the neurologic tumor was unaffected. Clearly, more patients and a longer follow-up are needed to clarify these preliminary impressions.

BCG given in this fashion is a simple and well-tolerated clinical procedure. It does not appear to be harmful to the patient, either directly or indirectly by enhancing tumor growth. The mechanisms of relapse in Burkitt's lymphoma are not presently known, although available evidence implicates the immune system. Further elucidation of these mechanisms, accompanied by refinements in immunotherapeutic approaches, may offer valuable adjuvant therapy in Burkitt's lymphoma.

Addendum: Since this paper was submitted for publication, a total of 33 evaluable patients have entered the trial. Seventeen patients received BCG and 16 were controls. Relapse occurred in 9 of 17 patients receiving BCG and 10 of 16 controls. The sites of relapse and the duration of remission were comparable in both groups, although an excess of patients with meningeal involvement as the sole manifestation of relapse was noted in the BCG-treated group. It can be concluded that while BCG exerts a potentiating effect on the cellular immune response, this form of immunotherapy failed to prevent relapse in Burkitt's lymphoma when given in the dosage and schedule employed in the trial. These results imply that impaired cell-mediated immunity is a result rather than a cause of tumor recurrence.

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DISCUSSION

R. L. Simmons: Dr. Ziegler, do you think there is no enhancement of normal relapse and that there is enhancement of neurologic relapse?

J. L. Ziegler: I pointed out the frequency of neurologic relapse in both groups is exactly the same: 3 in one and 4 in the other. It just seems that BCG prevents the emergence of systemic tumor but not the emergence of central nervous system damage.

S. R. Rosenthal: In the last few months I have been going to various centers that are using BCG. The difference in dosage has been enormous. Some centers have used a hundred times what the others have used and have obtained good results. So we may still have to do something about dosage.

E. Klein: I quite agree.

J. U. Gutterman: Dr. Sokal, was any of the patients receiving immunotherapy continuing chemotherapy?

J. E. Sokal: All patients were receiving maintenance chemotherapy. We did attempt to see if immunotherapy alone could carry any patient; to date only 1 patient has maintained a normal blood count for more than a year without any chemotherapy.

With the exception of this one rather unusual patient —who is now some 18 months since his last chemotherapy and has a white blood cell count of 5400—all other patients have had to have chemotherapy. Their chemotherapy has not been any different from that of our unimmunized patients, but did differ from the chemotherapy of the British patients.

J. E. Kennedy: Dr. Sokal, was there any way of distinguishing between tumor-specific antigens and HL-A antigens when the skin tests were evaluated?

Sokal: This is a complex problem. I don't know if we have tumor-specific antigens. We are using a crude homogenate of the cells in the skin test material, and how many antigens they contain I don't know. This is something that will require further immunologic study.

B. Zbar: Has your cell line been tested for the leukemia antigen that Dr. Mann has detected in other long-term lymphoid cell lines?

Sokal: No.

I. Djerassi: Dr. Ziegler, it must have been a very strange tumor if you can melt it with 1 or 2 shots of cyclophosphamide. Do you have any indication of what additional mechanisms other than chemotherapy may have been involved?

Ziegler: Yes. I should add to that list of immune mechanisms an observation of tumor-specific, cell-mediated immunity. Time did not allow a discussion of this, but we have identified in patients with Burkitt's lymphoma evidence of tumor-specific, cell-mediated delayed hypersensitivity. This also seems to correlate rather strongly with the clinical course of the patients, those having positive reactions demonstrating long clinical remissions.

We also noted that patients who tended to have relapses tended to be anergic both to recall skin tests and to the tumor-specific skin test. Hence we implemented BCG immunotherapy as a trial in these patients.

E. M. Pinsky: Dr. Sokal, you do not irradiate your cells?

Sokal: That is correct.

D. W. Weiss: Dr. Ziegler, I may have missed something in the design of this study, but, if you divide your groups between BCG-treated and nontreated patients on the basis of a starting population who respond well to cyclophosphamide, you may already be including good immune responders and you may not see a further difference due to BCG treatment.

Suppose you were to look at the results of 2 groups—BCG-treated and non-BCG-treated—both given cyclophosphamide therapy, but without the selection of those who are good cyclophosphamide responders. Would you then have a greater effect of BCG added to the cyclophosphamide schedule?

Ziegler: We have no way of knowing. We are obliged in these types of trials to ask only one question at a time. That would require a whole new study.

Weiss: You would be asking only one question at a time.

P. Alexander: Dr. Sokal, did you give chemotherapy weekly and the cell vaccine at the same time?

Sokal: Initially we were concerned about the possible antagonism between antileukemic chemotherapy and the immunizations. However, once the patient has his leukemia controlled and is on maintenance chemotherapy, the doses required to keep him in remission are relatively small and do not exert significant immunologic suppression. Therefore, we now ignore this factor completely; we just immunize when the patient is scheduled for immunization, although usually we ask him not to take his medicine on that day. But he takes medicine the day before or 2 days after.

S. D. Chaparas: Yesterday we were given some results indicating that we needed the organism in contact with

the cells to get remission. Dr. Ziegler, if you introduce the BCG intravenously rather than by scarification in the person who has immunity against tuberculosis and restrict the organism to the site so as to get into contact with leukemia cells, would you obtain better results?

Ziegler: I have no way of knowing. I can only say that the very patients we are treating are wondering why we do not use scarification on the tumor because that's what they are used to from their local witch-doctor practice in the countryside. The areas of pain or of tumor are scarified by the local practitioners, and herbs and other medicinals are put into these areas.

Unidentified speaker: Dr. Sokal, you used in your life table a 1-year lag time. Do you derive the 1 year from a preanalysis of the length of the survival of your patients in the immunotherapy series up to the time you started your life table (which is what I think would be done)? Or did you in fact derive the 1 year just out of the air?

Sokal: I reviewed the data, and it turned out that most of the immunized patients had had leukemia from about one-half year to 3 years when they were first immunized. The median was 19 months. So I figured any influence by using a year would be negative rather than positive. We used a year for convenience.

Immunoprophylaxis of Malignant Melanoma With Systemic BCG: Study of Strain, Dose, and Schedule[1,2]

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SUMMARY—Adjuvant immunotherapy with BCG was administered by scarification to 37 patients with advanced malignant melanoma who were clinically free of disease. A lyophilized strain of BCG (Tice) was compared to a liquid BCG (Pasteur) in various doses. High dose (6×10^7–6×10^8 viable organisms) Tice BCG was as effective as the Pasteur strain BCG in increasing the cellular immune response in melanoma patients. These preliminary results suggest that the treatment of malignant melanoma by a high dose of BCG, administered by scarification with an intensive schedule, may prolong the disease-free interval of melanoma.—Natl Cancer Inst Monogr 39: 205–212, 1973.

THE DEMONSTRATION of tumor-associated cell-surface antigens and specific immune responses to these antigens in both animal and human tumors has led to immunotherapeutic approaches for the control of a number of neoplasms (*1*). After the demonstration that BCG vaccine is a potent nonspecific immunostimulant in various animal tumor models (*2, 3*), clinical trials were conducted in leukemia (*4, 5*) and melanoma (*6*). Although encouraging, the initial clinical results have been empirical and arbitrary in regard to dose, route, schedule, and type (strain) of BCG preparation used. The objectives of this phase I study were:

1) To determine the efficacy of 2 strains of live BCG at various doses and schedules as immunoprophylactic agents in malignant melanoma.

2) To evaluate serially several immunologic parameters, including the primary immune response, established delayed hypersensitivity, in vitro lymphocyte blastogenic response to mito-

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gens and antigens, immunoglobulin levels, and lymphocyte and monocyte counts

In this report the initial early serial immunologic data are reported and are correlated with BCG dose clinical status. Definitive analysis must await longer follow-up of patients.

MATERIALS AND METHODS

Patient selection.—The criteria for entrance into the study were: malignant melanoma with no clinical evidence of disease but a high probability of systemic relapse. This included the following clinical situations:

1) Regional recurrence of melanoma totally removed by surgery: 17 patients (Stage III) (*7*).

2) Distance recurrence of melanoma totally removed by surgery: 12 patients (Stage IV) (*7*).

3) Chemotherapy-induced complete remission of disseminated metastatic disease: 3 patients (Stage IV) (*7*).

4) Local recurrence of melanoma totally removed by surgery: 2 patients (Stage II) (*7*).

5) Recurrent multiple primary melanomas: 3 patients (Stage I) (*7*).

Before immunotherapy, the patients were evaluated for metastatic or residual disease by the following tests: complete history and physical examination, complete blood count and differential, urinalysis, blood urea nitrogen, serum creatinine, serum transaminase, alkaline phosphatase, bilirubin, uric acid, calcium, lactate dehydrogenase, posterior-anterior and lateral chest X-ray, metastatic bone survey (including skull, lumbosacral, thoracic, cervical spine, and skull), liver scan, electroencephalogram, brain scan, bone marrow aspirate, and biopsy. In addition, most patients had whole lung tomograms. Informed consent was obtained.

Immunotherapy.—Fourteen patients were treated with lyophilized BCG from the Chicago Research Foundation (Tice strain). The BCG was administered by scarification with an 18-gauge needle applied on the upper arms or upper legs with 20 scratches, each 5 cm long, as described previously by Mathé et al. (*4*). Ten patients were treated according to schedule A: BCG weekly for 3 months, every other week for 3 months, and then monthly for 6 months. Six received a high dose, 6×10^8 viable organisms per scarification. Four received a low dose, 6×10^7 viable organisms per scarification (*see* table 1).

Four patients were treated according to schedule B: BCG twice weekly for 3 weeks and then monthly for 11 months. Two received a high dose, and two received a low dose.

Twenty-three patients were treated with fresh liquid BCG from the Pasteur Institute. All but 2 patients were treated according to schedule A (*see* table 2). Ten received the high dose of 6×10^8 viable organisms per scarification. Six received a middle dose of 6×10^7 viable organisms per scarification. Seven received a low dose of 6×10^6 viable organisms per scarification.

Immunologic evaluation.—Before BCG administration, skin tests with established delayed-hypersensitivity antigens were applied at least 7 days after the last surgical procedure or 30 days after the last dose of chemotherapy. The skin test antigens included dermatophytin, dermatophytin-O, and Candida antigen or Monilia mixture (all from Hollister-Steer Laboratories, Downers Grove, Ill. and all given in 0.1 ml of a 1/20 dilution of the stock), streptokinase-streptodornase (Varidase, Lederle Laboratories, Pearl River, N.Y., 0.1 ml, 50 U), mumps antigen (Lyovac-Mumpsvax, Merck, Sharpe & Dohme, West Point, Pa., 0.1 ml, 1/20 dilution of stock solution), and PPD (tuberculin purified protein derivative with Tween-80 preservative, intermediate test strength, Parke-Davis, Inc., Detroit, Mich., 0.1 ml). The antigens were injected intradermally on the forearm, and induration was measured at 24 and 48 hours. The average of 2 right angle measurements was recorded in millimeters. Skin tests were repeated montly for the first 3 months and every other month for the next 9.

Lymphocyte cultures.—These cultures were prepared as described in (*8*). They contained 10^6 lymphocytes, 1 ml of autologous serum, and 2 ml of minimal essential medium. Cultures were stimulated with the following: 0.05 ml of phytohemagglutinin-M (PHA, Difco Laboratories, Detroit, Mich), 300 μg keyhole limpet hemocyanin (KLH), 2000 μg streptokinase-streptodornase, 0.1 ml concanavalin A (Nutrional Biochemical Corporation, Cleveland, Ohio), 0.05 ml pokewood mitogen (Grand Island Biological Corporation, Grand Island, N.Y.), and 0.1 and 0.2 ml PPD (0.25 mg/ml). All cultures were harvested at 5 days, and the blastogenic response was measured by tritiated thymidine incorporation and recorded as counts per minute/10^6 lymphocytes. Lymphocytes were cultured weekly during the 1st month of therapy, every other week during the 2d month, and monthly thereafter (table 1).

KLH immunization.—Primary immunization with KLH was performed as described in (*9*). A total of 50 μg was given intradermally as the immunizing dose at the completion of the 2d week of BCG administration according to schedule A or on day 0 of schedule B. The skin test dose of KLH was 100 μg. The measurements were performed as described above. Skin tests were repeated 2, 6, and 10 weeks after immunization. The in vitro lymphocyte blastogenic response to the antigen was carried out as described above with 100 μg/ml of culture. Antibody titers to KLH were measured by passive hemagglutination with the use of chromic chloride-treated, KLH-coated red blood cells as the antigen (*9*). IgG antibody responses were determined by the resistance to 2-mercaptoethanol. The antibody titers were expressed as $\log_2$ of the highest positive serial twofold dilution. The antibodies were measured weekly after the immunization for the first 4 weeks, then every other week for the next 4 weeks, and once monthly thereafter.

Lymphocyte and monocyte count.—White blood cell counts with absolute lymphocyte and monocyte counts were done weekly for the first 4 weeks of treatment,

every other week for the next 4 weeks, and monthly thereafter.

Quantitative immunoglobulins.—The immunoglobulins were determined by quantitative immunodiffusion on serums drawn before study, every other week during the first 2 months, and monthly thereafter.

RESULTS

The clinical and immunologic data on the 37 patients are shown in tables 1 and 2. Of the patients treated with Tice BCG according to schedule A, 5 had regional recurrence, 4 had distant recurrence, and 1 had recurrent primary melanomas. There were 6 males and 4 females, and their median age was 49.5 years. Five of six receiving high-dose BCG and four of four receiving low-dose BCG remain in remission (median = 36 + wk).

Four males (median age = 58.5 yr) were treated according to schedule B. One patient receiving high-dose BCG and one patient receiving low-dose BCG have had relapses at 13 and 15 weeks, respectively.

High-dose Pasteur BCG was given to 6 males and 4 females (median age was 46 years). A total of 5 patients had regional recurrence, 3 had distant recurrence, 1 had local recurrence, and 1 had recurrent primary melanomas (table 2). Patient 19 was treated according to schedule B; the others all were treated according to schedule A. Of these 10 patients, 8 remain free of disease ranging from 12+ to 32+ weeks.

Middle-dose Pasteur BCG was given to 3 females and 3 males, whose median age was 56.5 years. Patient RA was treated according to schedule B; the others were treated according to schedule A. The patient treated according to schedule B and 1 patient with Stage IV disease have had relapses.

Low-dose Pasteur BCG was given 7 patients, 6 males and 1 female, whose median age was 45 years. Three had Stage III disease, three had Stage IV disease, and one had Stage II disease. All were treated according to schedule A. Of the 7 patients, 3 have had relapses. Two of the latter had Stage IV and one had Stage III disease.

The overall results correlating schedule, number of viable organisms per scarification, and stage of disease prior to therapy (table 3) are as follows: Of 17 patients with regional recurrence (Stage III), 14 remain in remission, with the median duration of remission 22+ weeks. Two of the patients having relapses were treated according to schedule B. Of 15 patients with Stage IV disease, 9 remain in remission with the median 24+ weeks. Of 24 patients receiving 6×10^7 or 6×10^8 organisms according to schedule A, 20 remain in remission. In contrast, only 4 of 7 receiving the lowest dose given according to schedule A (6 million organisms/scarification) remain in remission. Only 3 of 6 patients treated according to schedule B remain in remission.

The lung was the most common site of recurrence during immunoprophylaxis. One patient developed brain metastasis. Nine of the patients who have had relapses, including 6 with Stage IV disease, 2 with Stage III disease, and 1 with Stage II disease, are out of the study. Five of these patients have died, and four are still alive on chemotherapy.

The immunologic data are shown in tables 1, 2, and 4–6. Four of six patients receiving high-dose Tice BCG according to schedule A had increased numbers of positive skin tests after 12 weeks of immunoprophylaxis. Of 4 patients receiving low-dose Tice BCG according to schedule A, 3 also had an increased number of positive skin tests.

Five of seven patients in remission who received high-dose Pasteur BCG had an increased number of positive skin tests to established antigens. In contrast, only 1 of 4 patients receiving middle-dose and 2 of 4 patients receiving low-dose Pasteur BCG who remain in remission had an increased number of positive skin tests after 12 weeks of therapy.

The overall change in skin test reactivity to established antigens with Tice BCG treatment is shown in table 4. After 8 and 12 weeks of therapy, patients receiving high-dose Tice BCG had an overall median measurement of 10 mm for the 5 skin tests. In contrast, patients receiving low-dose Tice BCG did not show the overall increase in the median measurement of the 5 established skin tests. Of great interest was that, at 24 and 32 weeks, there was a gradual reduction in the overall measurement of 5 established skin tests in patients receiving high-dose Tice

TABLE 1.—Clinical and immunologic data on patients receiving Tice BCG

Patient	Age (yr)/Sex	Site of primary tumor	Site of recurrence	Stage*	Previous therapy	Reactivity to 5 established antigens before drug administered 12 weeks		Primary immunization with KLH†	Reactivity to PPD‡ before drug administered 12 weeks		Remission duration (wk)	Site of relapse outcome
Schedule A												
High dose:												
1	49/M	Trunk	Axilla	III	Surgery	2§	4	Yes	2‖	18	38+	—
2	45/M	Trunk	Axilla	III	Surgery	2	4	Yes	5	18	34+	—
3	53/F	Trunk	Axilla, trunk	IV	Surgery	2	3	Yes	0	10	46+	—
4	50/F	Face, foot	—	I	Surgery	2	3	Yes	0	20	30+	—
5	22/M	Trunk	Extremity	IV	Surgery	3	3	Yes	4	7	48+	—
6	62/M	Foot	Inguinal, iliac nodes	IV	Surgery	2	2	No	6	35	12	Lung, skin—dead
Low dose:												
7	69/F	Ear	Neck	III	Surgery	0	2	Yes	4	10	40+	—
8	34/M	Trunk	Axilla	III	Surgery	3	3	—	2	15	34+	—
9	35/M	Trunk	Axilla	III	Surgery	2	3	—	0	0	30+	—
10	66/F	Arm	Knee	IV	Surgery	0	1	Yes	0	9	38+	—
Schedule B												
High dose:												
11	33/M	Ear	Lung	IV	Surgery	2	4	Yes	5	18	34+	—
12	64/M	Ear	Neck, trunk	III	Surgery	2	3	No	0	8	13	Lung, bone—dead
Low dose:												
13	54/M	Trunk	Lung	IV	Surgery	1	3	Yes	4	17	15	Lung—dead
14	63/M	—	Lung	IV	Chemotherapy	2	2	Yes	6	18	34+	—

* *See* (7).
† Positive skin test 2–10 weeks after immunization.
‡ Intermediate test strength.
§ Number of positive (≥5 mm) skin test. *See* "Materials and Methods."
‖ mm.

TABLE 2.—Clinical and immunologic data on patients receiving Pasteur BCG

Patient	Age (yr)/Sex	Site of primary tumor	Site of recurrence	Stage*	Previous therapy	Reactivity to 5 established antigens before drug administered 12 weeks		Primary immunization with KLH†	Reactivity to PPD‡ before drug administered 12 weeks		Remission duration (wk)	Site of relapse outcome
High dose												
15	48/F	Trunk	Axilla	III	Surgery	0§	2	Yes	0	30	14+	—
16	18/F	Leg	Axilla	IV	Surgery	0	1	No	0	19	24+	—
17	33/M	Trunk	Axilla	III	Surgery	3	3	Yes	4	11	32+	—
18	23/M	Trunk	Axilla	III	Surgery	3	4	Yes	13	27	18+	—
19	62/M	Face	Submental	III	Surgery	2	3	No	0	20	25+	—
20	23/F	Trunk	Axilla	III	Surgery	4	3	Yes	0	30	18+	—
21	48/M	Trunk, arm	—	I	Surgery	3	4	Yes	19	15	28+	—
22	45/F	—	Lung	IV	Chemotherapy	—	—	—	—	—	12+	—
23	61/M	Nasal cavity	Nasal cavity	II	Surgery	2	2	Yes	3	19	20	Lung—on chemotherapy
24	47/M	Unknown	Neck	IV	Surgery	3	—	Yes	7	0	4	Liver, gastrointestinal tract—dead
Middle dose												
25	44/M	Trunk	Axilla	III	Surgery	0	4	No	0	16	13+	—
26	63/F	Left neck	Neck, skin	IV	Surgery	2	2	Yes	1	25	16	Skin—on chemotherapy
27	38/M	Trunk	—	I	Surgery	5	5	Yes	1	16	30+	—
28	70/F	Face	Neck	III	Surgery	3	2	Yes	18	16	14+	—
29	41/M	Neck	Lung	IV	Surgery	0	0	No	0	0	29+	—
30	69/F	Finger	Arm	III	Surgery	2	1	No	0	28	15	Lung—on chemotherapy
Low dose												
31	23/F	Trunk	Axilla, groin	IV	Surgery	2	2	Yes	0	0	24+	—
32	44/M	Trunk	Trunk	II	Surgery	0	1		0	10	20+	—
33	45/M	Finger	Axilla	III	Surgery	3	3	Yes	16	60	16+	—
34	55/M	Trunk	Axilla	III	Surgery	2	4	Yes	3	1	28+	—
35	51/M	Trunk	Axilla	III	Surgery	2	2	Yes	7	38	31	Eye—on BCG
36	74/M	Left leg	Right leg	IV	Surgery	2	3	Yes	12	20	12	Brain—dead
37	31/M	Trunk	Axilla, extremity	IV	Chemotherapy	0	0	No	0	23	12	Skin—on chemotherapy

* *See* (7).

† Positive skin test 2–10 weeks after immunization.

‡ Intermediate test strength.

§ Number of positive ($\geq$5 mm) skin test. *See* "Materials and Methods."

‖ Treated according to schedule B.

TABLE 3.—Correlation of dose and schedule of BCG with stage: Clinical results

Dose* and schedule	Stage I	II	III	IV	Total
			Schedule A		
6×10^8	1†/1	0/1	7/7	4/6	12/15
6×10^7	1/1	—	5/5	2/3	8/9
6×10^6	—	1/1	2/3	1/3	4/7
Total	2/2	1/2	14/15	7/12	24/31
			Schedule B		
6×10^8	1/1	—	0/1	1/1	2/3
6×10^7	—	—	0/1	1/2	1/3
Total	1/1	—	0/2	2/3	3/6

* Number of viable organisms administered per scarification.
† Number of patients in remission/total.

TABLE 4.—Skin test reactivity to established antigens with Tice BCG treatment

	Weeks after BCG started 0*	4	8	12	24	32
High	0*	5	10	10	7	3.5
Low	0	1	0	1	0	0
Relapse	3	5	5	—	—	—

* Median measurement (in mm) of 5 skin tests.

TABLE 5.—Skin test reactivity to established antigens with Pasteur BCG treatment

	Weeks after BCG started 0	4	8	12
High	0*	0	0	5
Mid	0	0	5	5
Low	0	1	2	1
Relapse	2	0	0	—

* Median measurement (in mm) of 5 skin tests.

BCG, which correlated with the decrease in the frequency of BCG scarification.

In table 5 is shown the change in skin test reactivity in patients receiving Pasteur BCG. Patients receiving high and middle doses had an increase to 5 mm at 12 weeks, which was not as great as the increase seen in patients receiving high-dose Tice BCG.

Eight patients were anergic to the battery of 5 established antigens and PPD before immunoprophylaxis (table 6). Of the 8 anergic patients, 6 have converted ≥ 1 positive established skin-test antigen and PPD to positive and 7 of these patients remain in remission from 19+ to 40+ weeks (as of this report).

TABLE 6.—Immunoprophylaxis of malignant melanoma: Prognosis of anergic patients

BCG dose	Number anergic	Number converted	Number having remission	Median duration (wk)
		Tice		
Low	2	2	2	40+
High	0	—	—	—
		Pasteur		
Low	2	1	1	20+
Middle	2	1	2	21+
High	2	2	2	19+
Total	8	6	7	

As shown in tables 1 and 2, 7/9 patients receiving Tice BCG who remain in remission were successfully immunized with KLH. In contrast, only 1 of 3 patients who subsequently had relapses was successfully immunized. Ten of fourteen Pasteur-treated patients in remission and five of seven Pasteur relapse patients were successfully immunized with KLH. Antibody response was slightly greater at 12 weeks in Tice BCG-treated patients in remission compared to the Pasteur BCG-treated patients in remission. At 12 weeks, the median antibody titer ($\log_2$ total/2-mercaptoethanol resistant) was 9/7 for

the Tice BCG-treated group and 7/4 for the Pasteur BCG-treated group.

Before BCG administration, there was no significant difference in the skin-test response to PPD of patients who were still in remission compared to patients who had subsequent relapses. Fifteen of 19 patients negative to PPD before therapy, who remained in remission, converted the PPD skin test. All 6 patients negative to PPD, who subsequently had relapses, converted the PPD skin test.

There was no overall change in the vitro lymphocyte blastogenic response to phytohemagglutinin, pokeweed mitogen, streptolysin-O, concanavalin A, or streptokinase-streptodornase.

During therapy, there was no overall change in the absolute lymphocyte and monocyte counts nor was there a significant change in the immunoglobulin levels.

Scarification was well tolerated by all patients. About 40% of patients experienced fever, 101° F, with mild malaise starting 24 hours after scarification and lasting 24–48 hours. Frequently there was increased heat with edema, and itching occurred over the site of the most recent vaccination as well as other scarification sites. Focal infection at the site of scarification did not develop. Liver abnormalities and disseminated BCG disease were not observed in any patient.

DISCUSSION

The initial report by Mathé et al. suggesting a beneficial effect of BCG vaccination in the treatment of acute lymphoblastic leukemia (*4*) has stimulated additional trials with systemic nonspecific immunotherapy in human malignancies. Recently, Bluming and co-workers presented data suggesting that Pasteur BCG given by scarification prolongs disease-free intervals in malignant melanoma (*6*). In contrast, the intradermal vaccination with Glaxo BCG was associated with an early relapse rate. However, as the authors point out, the latter treatment may have adversely affected those patients. The British used the Glaxo strain of BCG (administered intradermally) in doses of < 1 mg per week and failed to confirm any therapeutic benefit of BCG in acute lymphoblastic leukemia (*10*). From these studies and also from work done in animals suggesting that different strains of BCG have a varying therapeutic efficacy (*11*), it appeared to us that a further evaluation of dose, route, schedule, and type of BCG preparation was required to design optimal programs of nonspecific immunotherapy in man.

This preliminary analysis of the immunoprophylaxis of patients with recurrent melanoma suggests that the higher doses of BCG (at least 6×10^7 viable organisms) given according to the more intensive schedule may be associated with a better prognosis than the administration of lower numbers of viable BCG organisms. Thus only 4 of 24 patients receiving 60 or 600 million viable organisms by the intensive schedule have had recurrence of disease. In contrast, 6 of 13 patients receiving 6 million BCG organisms or receiving BCG according to the less intensive schedule have had recurrence of disease.

In particular, patients with regional lymph node metastasis have thus far done well. In our experience, 40% of patients with regional lymph node recurrence have had further recurrence of the disease in the 1st year of follow-up (C. McBride, unpublished data). The length of follow-up of this study is still too early to determine if this particular group of patients will have an improved prognosis with BCG treatment. As expected, the patients who have Stage IV disease removed by surgery before they entered this study have not done as well as patients with Stage III disease. These patients have blood-borne metastasis, and their recurrence rate in the 1st year after surgery is 80% (C. McBride, unpublished data). Once again, this study is too early to ascertain any therapeutic benefit with BCG. (*See* note added in proof.)

The current study to date, therefore, suggests that patients receiving lyophilized Tice BCG have done as well as, of not better than, those receiving the liquid strain of Pasteur BCG. We suggest that the most important variable in such a BCG trial is the actual number of viable organisms administered rather than the strain of BCG used. This is important because the short shelf life of liquid Pasteur BCG makes it rather impractical to use in this country compared to using a freeze-dried preparation. It is likely that patients treated with Pasteur BCG have received fewer viable organisms than actually prescribed.

Patients receiving high-dose Tice BCG have had greater increases in skin test reactivity compared to patients receiving low-dose Tice BCG. Thus far, however, there has been no difference in the clinical course between these 2 groups of patients. Patients receiving high- and middle-dose Pasteur BCG had greater increased skin-test reactivity compared to patients receiving low-dose Pasteur BCG. Of great interest is that patients receiving lyophilized Tice BCG had greater increases in skin-test reactivity compared to patients receiving high-dose Pasteur BCG.

Another point of interest is that 7 of 8 anergic patients converted to positive at least one skin-test antigen in addition to PPD. This is of great importance, since these patients generally have poor prognosis (*12*). Seven of the eight patients are still in remission, and there was no morbidity associated with the BCG.

Similar to the report by Bluming et al. (*6*), there was no change in the lymphocyte counts or immunoglobulin levels during the study. In addition, there was no change in the lymphocyte response to the variety of mitogens or antigens. Jones et al. (*13*) recently showed no change in lymphocyte reactivity to phytohemagglutinin with BCG treatment in a group of patients with acute leukemia. Perhaps using lower doses of mitogens such as reported recently by Faguet et al. (*14*) will detect changes in lymphocyte reactivity in vitro.

In conclusion, these results suggest that high-dose Tice BCG is as effective as Pasteur BCG in increasing the cellular immune response in melanoma patients. These preliminary results also suggest that the immunoprophylaxis of malignant melanoma by a high dose of BCG, administered by scarification with an intensive schedule, may prolong the disease-free interval and/or survival of these patients.

Note added in proof: An updated, definitive analysis, suggesting a prolongation of disease-free interval and survival in patients with resectable advanced malignant melanoma by treatment with lyophilized Tice BCG compared to liquid Pasteur BCG, has been published by us in Lancet 1:1208–1212, 1973.

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Immunotherapy of Melanoma With Intralesional BCG[1, 2]

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SUMMARY—A total of 19 patients with histologically confirmed recurrent melanoma were treated with intralesional BCG. Of the 15 evaluable patients, 5 had clinically significant responses. One patient had a complete remission with response in both injected and uninjected lesions. Four other patients had partial remissions, including 3 still under treatment. Clinical response was associated with a small, local tumor burden and the virtual absence of any serum blocking effect. All 4 patients with purely intradermal disease responded; patients with subcutaneous and/or visceral disease failed to respond and, in some cases, may have had a BCG-induced facilitation of tumor growth. In the latter cases, serum blocking activity was generally prominent.—Natl Cancer Inst Monogr 39: 213–220, 1973.

EXTENSIVE INVESTIGATION of the immunology of animal and human neoplasia in recent years suggests that host immunity is a significant factor in the host resistance to cancer and is capable of augmentation (*1, 2*). Encouraging results have come from several previous studies in which intralesional BCG was used to augment immunity in melanoma patients (*3–5*). This report summarizes our experience with intralesional BCG in 19 patients with histologically confirmed recurrent melanoma (*6*).

MATERIALS AND METHODS

The major objectives of our study were: 1) to delineate the melanoma patient population that might benefit from intralesional BCG treatment; 2) to serially evaluate the immune response to treatment by in vitro techniques; and 3) to define any adverse effects of the treatment. Before treatment, each patient was evaluated to define the mass and distribution of disease. The immune status of the patient was also studied, using dinitrochlorobenzene sensitization, a battery of microbial extracts, typhoid vaccination, and protein electrophoresis. Fibroblast cultures from skin and tumor cultures were established from each patient before treatment. Blood for serial in vitro tests was collected from each patient before treatment and at each clinic visit thereafter. In vitro studies included tests of lymphocyte cytotoxicity, comple-

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ment-dependent serum cytotoxicity, and serum blocking activity.

One-tenth of a milliliter of BCG (obtained from the Research Foundation, Chicago, Ill.) was injected intradermally (id) into a single lesion. Injections were repeated biweekly for a minimum of 5 injections, which we defined as an adequate trial of immunotherapy. The injected dose was increased tenfold when no response followed. A separate lesion was injected at each visit when possible. The first 3 patients, however, received variable doses of BCG, constituting a feasibility study for our protocol design.

Of a total of 19 patients in our study, 4 were unevaluable for response, in that they received only 1 or 2 injections. Of these 4 patients, 3 died of advanced disease early in treatment. The fourth patient refused treatment after 2 injections because of mild toxicity. This patient had previously abandoned chemotherapy for a similar reason.

RESULTS

Of the 15 patients evaluable for response, we observed 5 clinically significant responses. One patient with recurrent id lesions had a complete remission. All injected lesions, and some uninjected lesions, regressed. Regression was associated with a local inflammatory reaction. She is now 18 months since the start of immunotherapy and has been in complete remission, without treatment for 10 months. The clinical course of this patient will be detailed later.

Three other patients with id disease are now under treatment and to date have had partial remissions. One of these patients had the regression of uninjected lesions without the usual inflammatory response (figs. 1–4). These lesions became flat and lighter in color. When biopsied, they were found to have a marked infiltration of melanin-filled histiocytes and lymphocytes (under light microscopy) and no tumor cells. Granulomas were also prominent. These findings are similar to those recently reported in a guinea pig tumor system following BCG injection (*7*).

A fifth patient with both id and subcutaneous (sc) disease had a partial remission. Both the id and sc lesions regressed, but this patient now has progressive disease.

In our series, patients with only sc and/or visceral metastases failed to respond to this form of immunotherapy. Furthermore, in several instances, the disease progressed markedly after the start of BCG administration. This rapid acceleration may represent the natural history of the disease in these patients, but the possibility that BCG stimulated tumor growth must be considered. This phenomenon is not without precedence in certain animal tumor systems (*8–10*).

Patients were clinically staged according to the extent of disease. Eleven patients had regional disease, confined to one anatomic region with or without regional lymph-node involvement. Of these patients, 4 had only id disease and all were responders. One further patient had both sc and id disease and also had a partial remission. The other 6 patients had sc and/or lymph node involvement and all failed to respond. Four patients had disseminated disease, 3 of whom had visceral involvement. None responded to treatment.

It is difficult to appraise total tumor burden, particularly in those patients with visceral metastasis. However, estimates of body tumor burden made periodically on all patients revealed that responders apparently had somewhat smaller tumors than nonresponders ($P = 0.10$, Mann-Whitney U Test) (*11*).

Systemic toxicity occurred in most patients, although it was not a dose-limiting factor. Most prominent was fever, often up to 104° F, beginning several hours after the injection of BCG, and generally subsiding with 36–48 hours. Chills, anorexia, nausea, occasional vomiting, and generalized malaise also often occurred. Patients infrequently complained of myalgias and arthralgias. Occasionally, both the malaise and fever persisted for 7–10 days. No clinical evidence of disseminated tuberculosis was noted, nor were any patients treated with antituberculosis medications.

The results from completed analysis of our in vitro studies will be published in the future. Preliminary evaluation has thus far suggested the following tentative conclusions:

Those patients whose disease may have been accelerated by BCG therapy tended to show a pronounced blocking activity, often before or early in the course treatment. In contrast, the responders had little or no blocking activity in their sera. Both groups of patients generally

had cytoxic lymphocytes. Cytoxicity may actually have been weaker in the responders than in those whose disease progressed rapidly.

The clinical response of our longest responder is of interest. A 45-year-old female, she had a primary melanoma which had been removed from the anterior tibial aspect of the right leg in August 1969. In June 1970, a local recurrence was widely excised, and a groin dissection revealed 1 of 7 lymph nodes to be positive for melanoma. In October 1970, the patient presented with recurrent satellite lesions and was treated in succession with bleomycin, 1,3-bis (2-chlorethyl) -1-nitrosourea, and imidazole carboxamide; the disease progressed. In April 1971, two months after the last chemotherapy, immunotherapy was begun. She had a complete remission and was last treated in November 1971 (figs. 5–10).

Our observations are consistent with the hypothesis that immunotherapy can be beneficial in treating recurrent human melanoma. However, the approach remains investigational and only through carefully conducted trials can we delineate the proper dose, route, frequency, and type of immunotherapy and establish guidelines for proper patient selection for this form of treatment.

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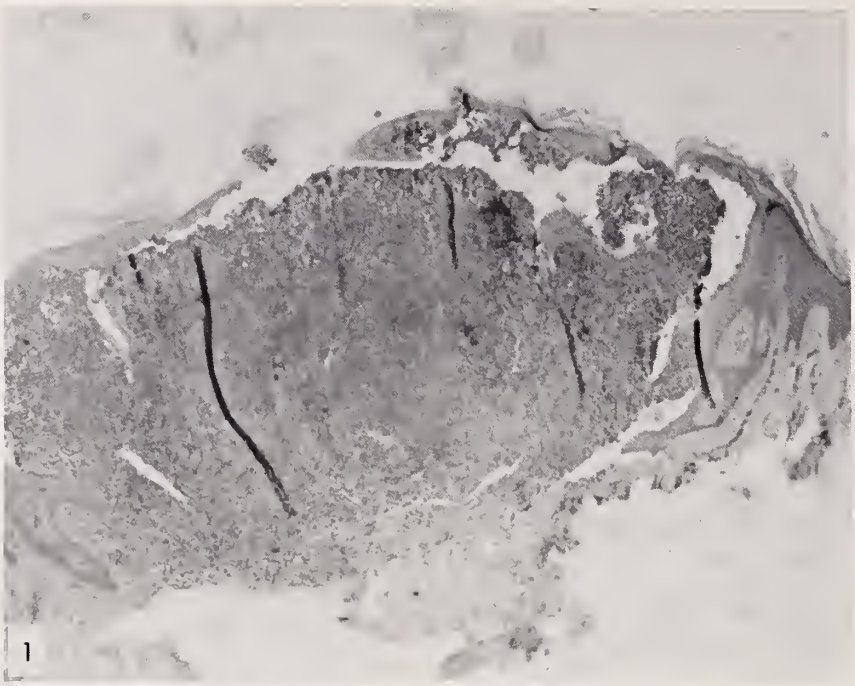

FIGURE 1.—Pretreatment biopsy showing elevation of epidermis and infiltration of tumor cells.

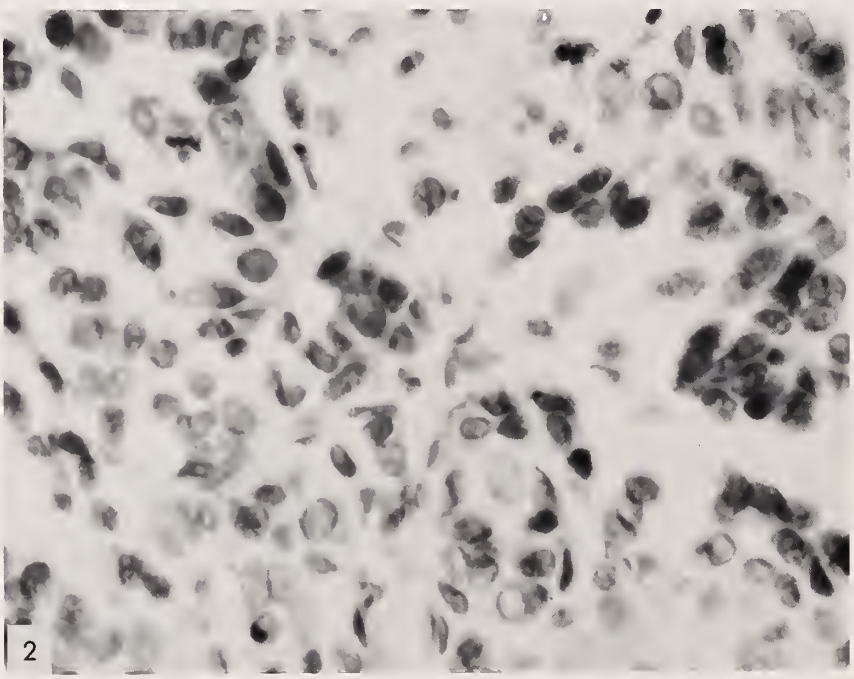

FIGURE 2.—Pretreatment biopsy showing infiltration of tumor cells. × 40

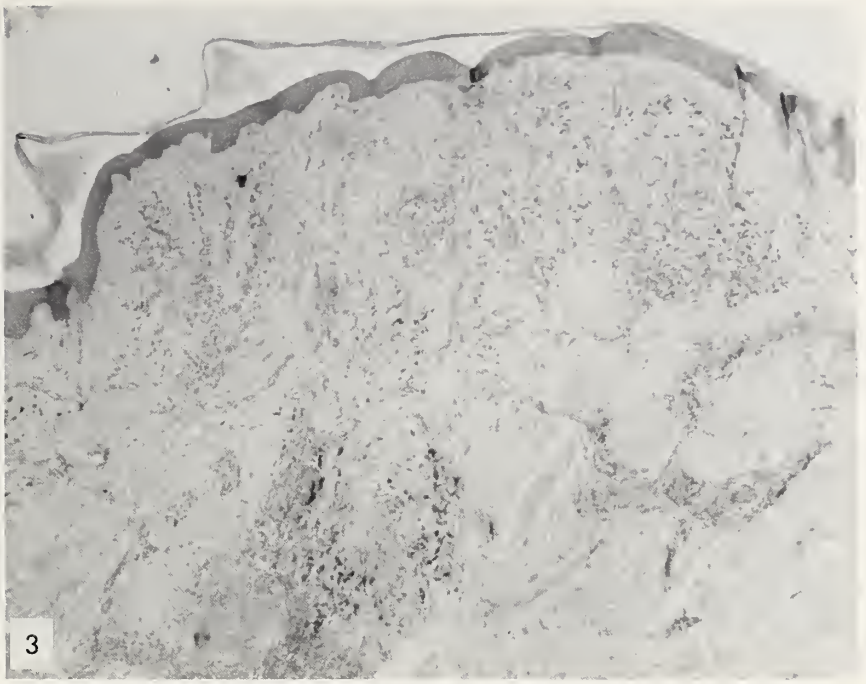

FIGURE 3.—Post-treatment biopsy of regressing, uninjected lesion showing elevation of epidermis and cellular infiltration.

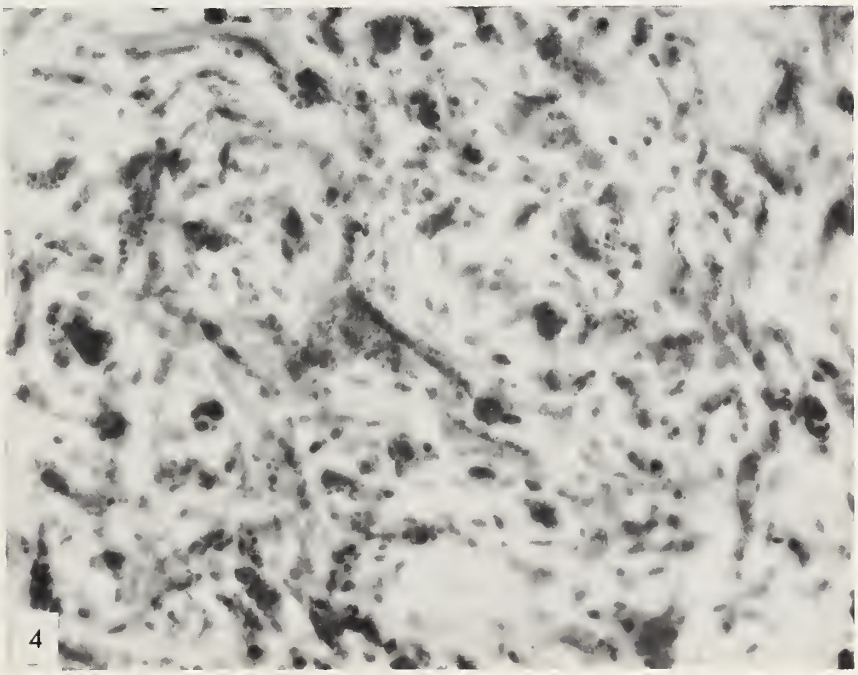

FIGURE 4.—Post-treatment biopsy of uninjected lesion showing melanin-filled histiocytes and lymphocytes with absence of tumor cells. × 40

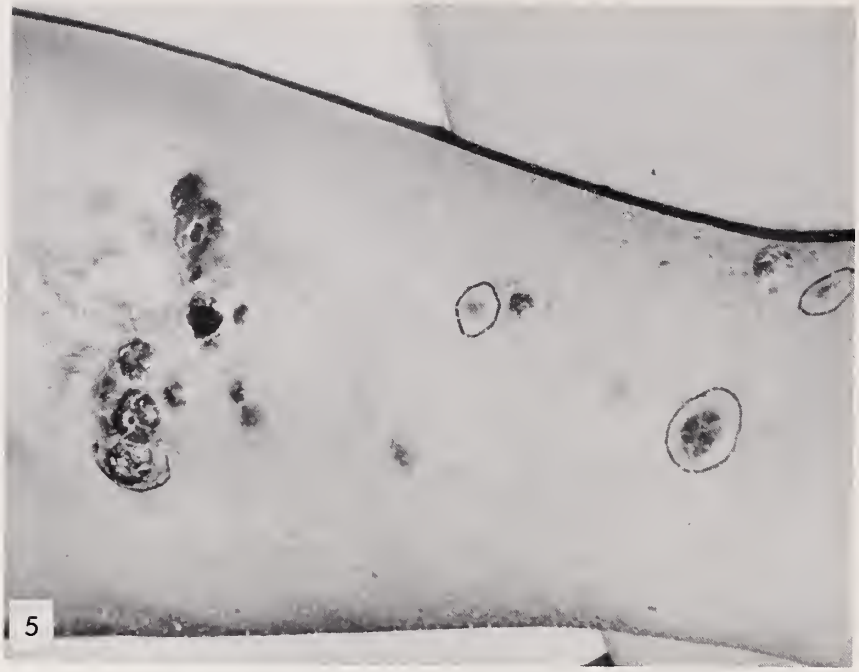

FIGURE 5.—Intradermal lesions before treatment.

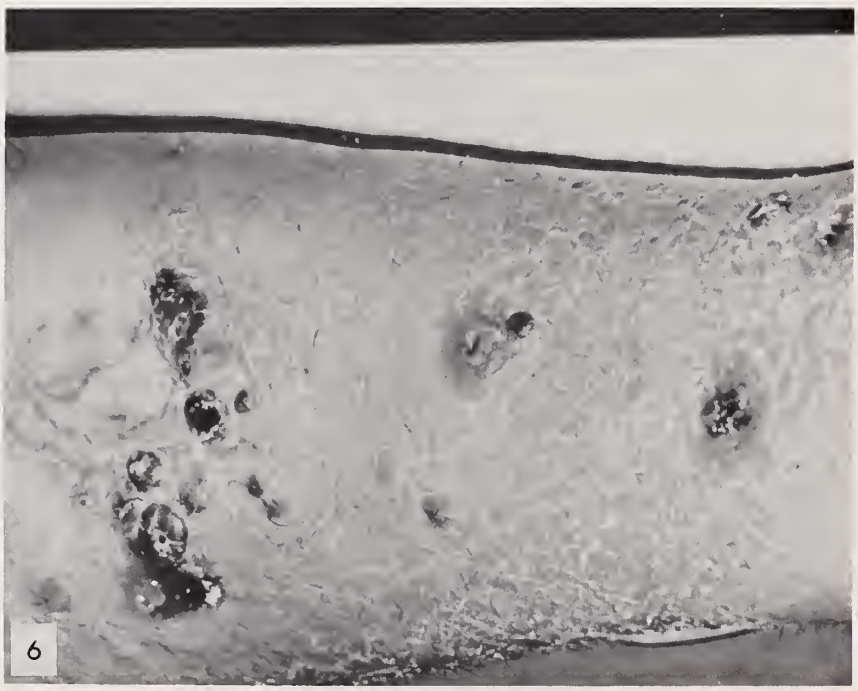

FIGURE 6.—Local reaction 2 weeks after BCG injection.

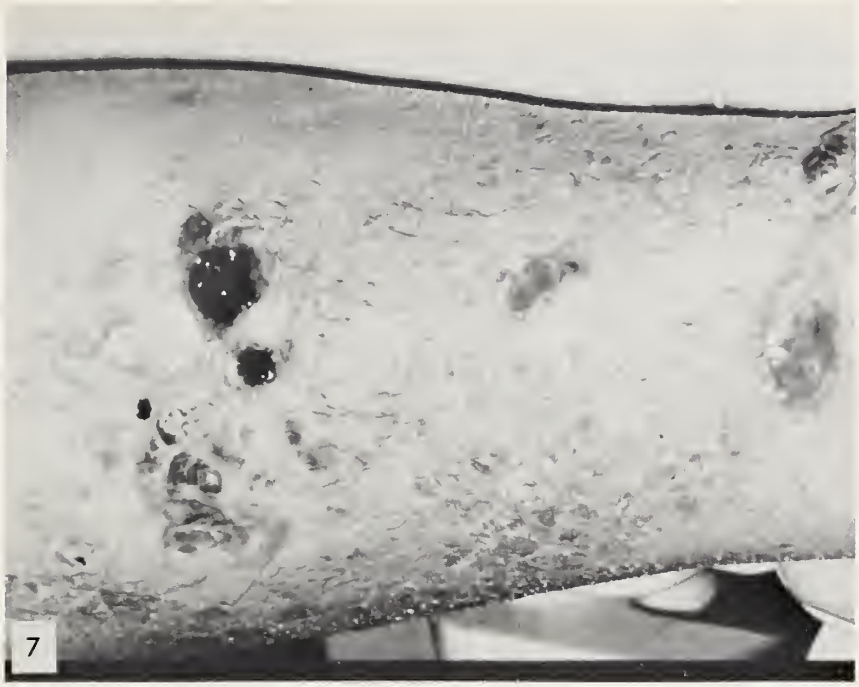

FIGURE 7.—Six weeks post injection. Ink spot (left) shows site of second injection.

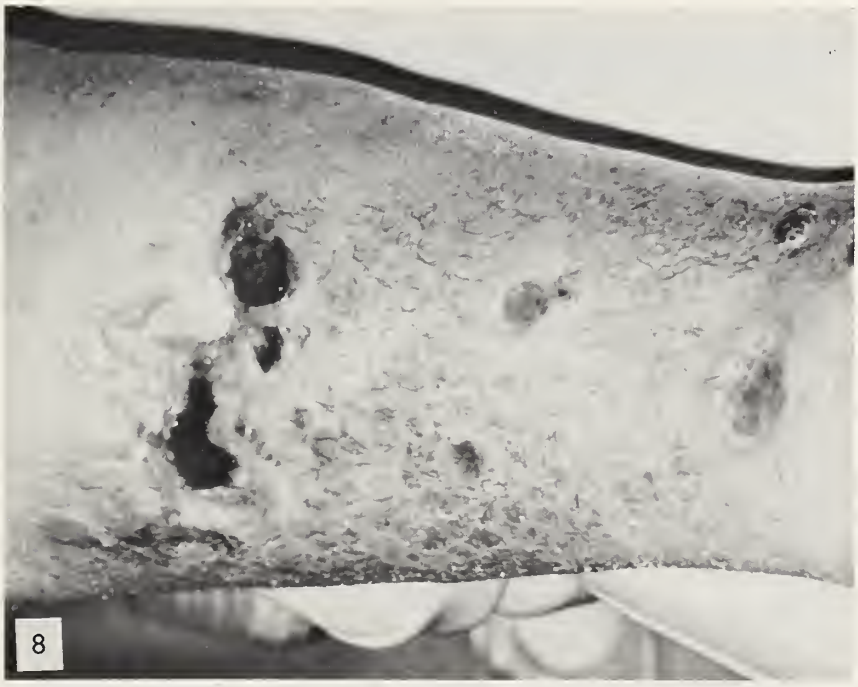

FIGURE 8.—Seven weeks post injection. Uninjected lesion in *lower* left shows inflammation.

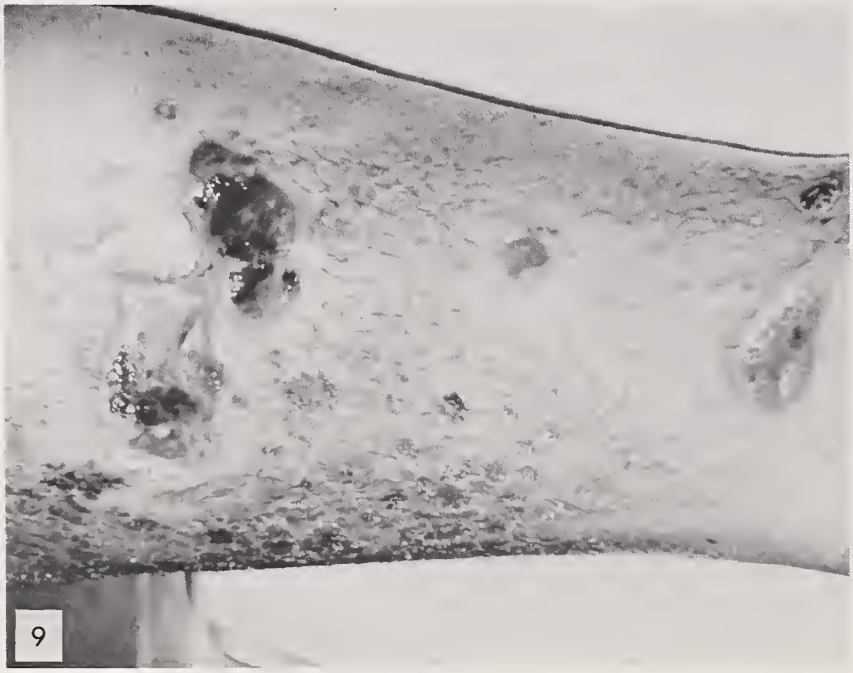

FIGURE 9.—Nine weeks post injection. Uninjected lesion in *upper* left shows inflammation.

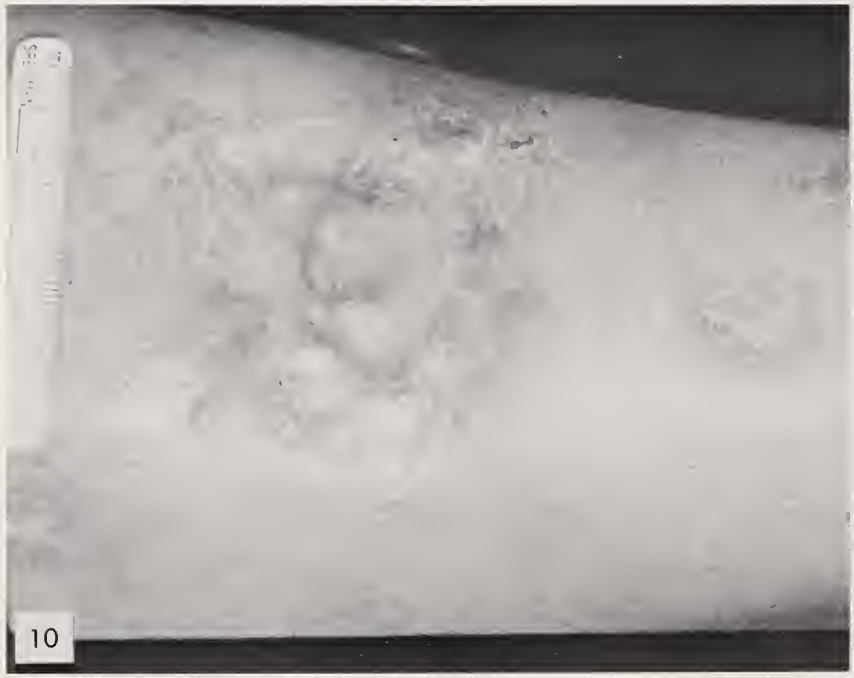

FIGURE 10.—Eighteen months post injection. No evidence of tumor.

In Vitro Assay of Cell-Mediated Immunity in BCG Therapy of Malignant Melanoma: A Preliminary Report[1, 2]

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SUMMARY—Preliminary data suggest that BCG-induced tumor regression is accompanied by an increase in lymphocytoxicity demonstrated by an in vitro assay. "Blocking activity" was never observed in the regressing stage. A patient with metastatic melanoma, who was treated with a combination of BCG and a cytotoxic agent (dimethyltriazenoimidazole carboxamide) and is still in complete remission > 2 years after onset of therapy, showed similar serial changes in lymphocytotoxicity and blocking.—Natl Cancer Inst Monogr 39: 221–224, 1973.

SEVERAL REPORTS have provided evidence of clinical improvement in human malignant melanoma with BCG therapy. BCG therapy has included intralesional BCG injection (*1–4*), intradermal BCG or BCG scarification (*5*), and injection of BCG mixed with either sensitized lymphocytes or autologous irradiated tumor cells (*6, 7*). These papers have yielded evidence suggesting that an immunologic mechanism was responsible for the beneficial effects observed. However, in none of these reports did all patients so treated achieve improvement. To elucidate the reasons for this differential effect, the immunologic status of patients must be monitored at the same time as the patients are undergoing therapy. Several techniques have been used to measure cellular immunity to antigens unrelated to the tumor under treatment. However, assessment of cellular immunity specifically related to tumor antigens has been attempted in relatively few studies and has included the use of the mixed lymphocyte reaction (*8*) and lymphocytotoxicity assays (*3, 9*). The following report deals with our preliminary attempts to develop an in vitro assay system for monitoring tumor-specific immunologic changes occurring in these patients before, during, and after treatment with BCG.

MATERIALS AND METHODS

Patients.—Patients who had intradermal metastases from malignant melanoma without clinical evidence of systemic disease were treated with up to 8 intralesional doses of BCG obtained from the Tice Laboratory of the University of Illinois. This BCG strain originated in the Pasteur Institute and contains approximately 3×10^7 colony-forming units/cc. Approximately 0.1 cc of solution was injected per lesion, with up to 6 lesions being injected at

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a given time. Some nodules were left uninjected, and others were injected with saline to serve as controls. Patients having tumor nodules biopsied before and during regression comprised the "therapeutic" group. An additional group of patients in whom all metastatic disease had been surgically removed constituted the "prophylactic" group. All of these patients had an overwhelming clinical likelihood of developing recurrent disease; to these patients we administered BCG for 6 weekly intradermal doses, rotating the deltoid and gluteal areas.

In vitro lymphocytotoxicity.—The method used, described in (*10*), will be briefly reviewed. Tumor cells, obtained by excision of metastatic nodules, were grown in monolayer in Ham's F-10 medium with 20% fetal calf serum (FCS). After several tumor cell subcultures, pleuropneumonia-like organisms and bacterial cultures were done, and appropriate antibiotics were used to suppress any organisms demonstrated. The lines were identified as melanoma by their aneuploid karyotype, by their positive staining with 3,4-dihydroxyphenylalanine oxidase, by electron microscopic demonstration of intracellular inclusions, and by their heterotransplantation into antilymphocyte serum-immunosuppressed, neonatal, Wistar-Furth rats that showed growth of typical pigmented tumors. Tumor cells were harvested from monolayer by trypsinization (0.025%) and suspended in media with 20% heat-inactivated FCS. Since some lots of FCS are either immunosuppressive or may have some sort of leukoagglutinins, which may render lymphocytes cytotoxic, it is desirable to obtain samples of serial lots, test them, and then use the optimal lots which are stored at −20° C. A total of 50–100 tumor cells were seeded per well in 0.2-ml volumes in Linbro Disposo-trays with 16-mm wells. The following day, the media were decanted; 0.2 ml of 1/6 diluted heat-inactivated (56° C for 30 min) sera was added after filtration through 0.22μ Millipore filters. All of these manipulations were carried out in a laminar flow hood, and the sera were added in French square fashion in the plates to insure representation on every plate of any given serum. The mixture was diluted 1/6 in 0.01 M phosphate-buffered saline, pH 6.8. After 45–60 minutes' incubation in 5% CO_2 at 37° C, the test sera were decanted, and 0.5×10^6 lymphocytes in 0.5 ml with F-10 media were added.

Lymphocytes were obtained by Wood's method, a technically simple, highly reproducible technique which appears to work well. Attempts to use the Ficoll-Hypaque technique on defibrinated blood seemed less successful. In the Wood's method, plasmagel-sedimented supernatant from lightly heparinized blood is defibrinated with a mixture of nylon powder and glass beads. An additional advantage of the Wood's technique appears to be that lymphocytes can survive freezing and subsequent washings.

Each combination of sera and lymphocytes had at least 6 and frequently 8 replicates. All sets of lymphocytes were HL-A typed before use to demonstrate histocompatibility. Plates were placed on top of the rocker for 45–60 minutes, and 0.5 ml 40% FCS was added. Preparations ranged from 95 to 99% lymphocytic purity and were >90% viable as judged by the trypan-blue exclusion technique. Rocking was terminated at 48 hours, and the plates were washed with saline, fixed with ethanol, and stained with Giemsa. Cell-mediated immunity was reflected by a reduced tumor-cell count in wells exposed to immune as contrasted to control lymphocytes, and "blocking activity" was reflected by the abrogation of this effect with appropriate sera. Inhibition of lymphocytotoxicity with mitomycin C excluded allogeneic and agglutination cytotoxicity as mechanisms for target cell damage. ABO and HL-A compatibility of the tumor cell donor, test sera, and lymphocytes was established. All washings were carried out gently so as to dislodge all red blood cells and dilute FCS without disturbing target tumor cells. After 2 washes, 95% ethanol was added, left for at least 10 minutes, and decanted. Plates were then washed in distilled water and dried. Giemsa staining for 15 minutes was followed by 1 wash with distilled water.

RESULTS

Table 1 summarizes data on lymphocytotoxicity in patients undergoing therapy with BCG. In the pretreatment state, all patients had progressive disease; "regressors" are those patients destined to have regressing tumors after BCG treatment. Pretreatment samples were obtained before the clinical status was altered by BCG therapy. Post-treatment samples were taken about 2 weeks after such alterations were evident. Among the responding patients are two who still have no clinical disease, one 14 and one 25 months after onset of BCG therapy. In "prophylactic" patients, regression was defined as the absence of recurrent disease, whereas progression was defined as the presence of such disease. Although the number of observations was small, there appeared to be some significant differences. Patients who showed tumor regression following BCG treatment also developed rising lymphocytotoxicity, whereas those in the "progressor" state did not. The most consistent finding in the prophylactic group was that patients with early recurrence, most of whom had relapses with visceral metastases, did not have rising lymphocytotoxicity after intradermal administration of BCG. Prophylactic treatment with BCG was associated with greater lymphocytotoxicity in patients demonstrating no early recurrence. However, data are incomplete in the pretreatment state, and follow-up is still <2 years.

TABLE 1.—Lymphocytotoxicity in patients undergoing BCG treatment of melanoma

	Therapeutic		Prophylactic	
	Before	After	Before	After
Regressors	23% (3)*	59% (5)		50% (2)
Progressors	17% (4)	24% (7)		17% (1)

* Numbers in parentheses indicate number of patients.

TABLE 2.—"Blocking activity" in BCG treatment of melanoma

	Therapeutic		Prophylactic	
	Before	After	Before	After
Regressors	1/2*	0/5		
Progressors	2/2	5/2		

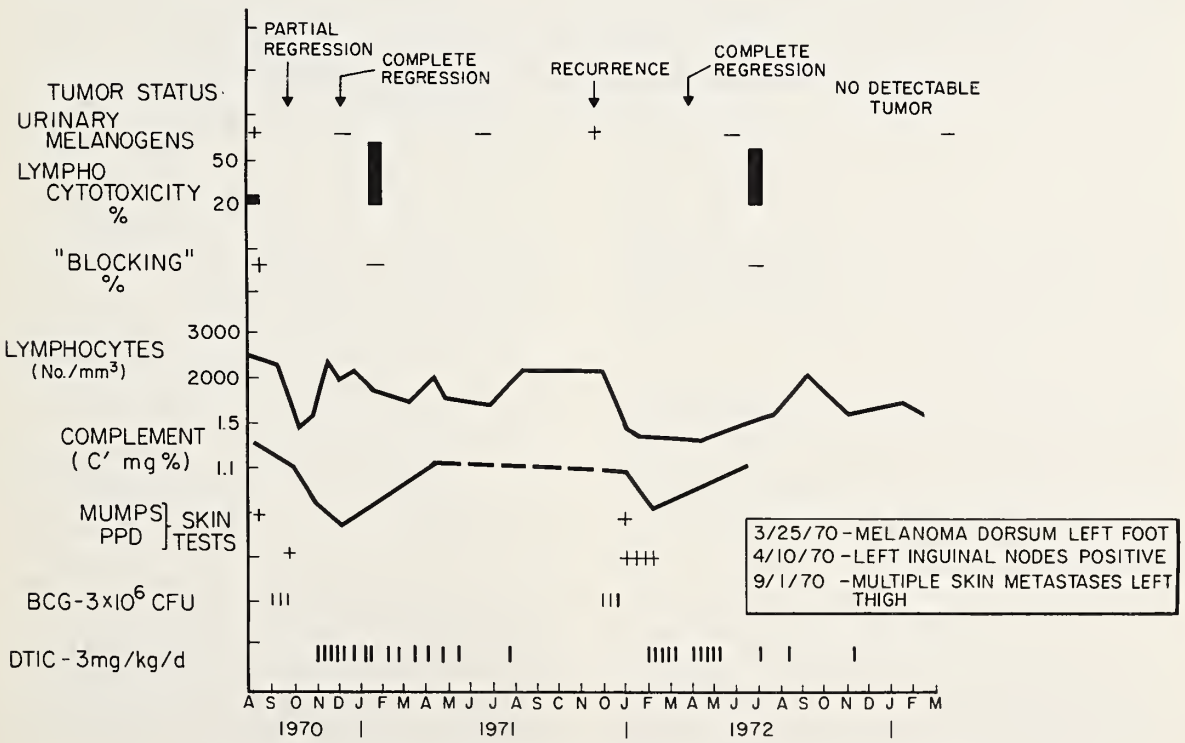
* Number positive/No. negative or absent.

Table 2 shows the results with "blocking activity" data in the "therapeutic" group. Almost all patients with failure on BCG therapy demonstrated blocking. Of those who did not, an immunologically privileged site of metastasis, such as the brain, may have accounted for this failure. No patients with BCG-associated tumor regression demonstrated blocking.

Text-figure 1 shows the serial data on a patient who exhibited an initial BCG response, was then treated with a cytotoxic drug, dimethyltriazenoimidazole carboxamide (DTIC), and went on to develop a complete response. She had a recurrence of tumor about 14 months after initial treatment but then developed a second complete tumor remission. Now 25 months post treatment, she is completely free of any known tumor. Of interest is the consistent and significant drop in β_1C complement component and lymphocyte levels, following BCG treatment. She demonstrated a striking increase in lymphocytotoxicity and absent blocking with her BCG–DTIC-induced remission. Her positive urinary melanogens became negative, which suggested visceral and intradermal tumor regression (*11*).

DISCUSSION

These data, although preliminary, are consistent with the possibility that BCG in some



TEXT-FIGURE 1.—Immuno-chemotherapy data on a female patient with melanoma.

way augments cell-mediated immunity in the host specifically directed at tumor antigen. Since BCG is a nonspecific stimulant of antibody formation (*12*) and of cellular immunity (*13*), this observation seems consistent with a body of data in the treatment of experimental tumors in animals (*14*). Alexander et al. (*15*) studied limb tumors in animals. They suggested that a defect in the afferent immune pathway renders the draining nodes incapable of producing specific lymphoblasts. This defect is only in the nodes draining the tumor and is specific for the tumor antigen. BCG could counteract this afferent defect by bringing additional nodal structures into active participation in the immune response. More recently, Evans and Alexander (*16*) demostrated the "killer-cell" effect of macrophages. This work suggests another role for BCG, that of macrophage mobilization or activation. The possibility of this stimulus to the efferent immune pathway seems consistent with other data demonstrating that BCG may enhance phagocytic function (*17*).

The use of combinations of drugs and immunologic adjuvants together is not new. In Fefer's Moloney sarcoma virus model (*18*), it is of interest that cytotoxic drugs combined with immune spleen cells could produce long survival not seen with either modality alone. In our patient, BCG could have induced proliferation, activation, or mobilization of specifically sensitized immune competent cells in a fashion quite analogous to that of Fefer's exogenously administered immune spleen cells. The falling peripheral lymphocyte count after BCG treatment in this patient and other patients may be consistent with this hypothesis.

Addendum: The patient mentioned in text-figure 1 is still alive but has just relapsed for a second time (October, 1973), 38 months after start of therapy. Her sensitivity to BCG precludes further use and she is now on DTIC.

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Treatment of Malignant Melanoma by Intratumoral Injection of BCG[1, 2]

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SUMMARY—Twenty-nine patients with metastatic malignant melanoma were treated by the injection of BCG into cutaneous lesions. Inflammation and ulceration of injected nodules occurred in 21 cases and subsequent regression of these tumors in 10. Noninjected cutaneous lesions simultaneously regressed in 2 patients, one of whom has been free of disease for 22 months. Conversion to a positive tuberculin test occurred in 10 of 12 patients positive to 2, 4-dinitrochlorobenzene. While BCG bacteremia was observed only up to 5 minutes after injection of the BCG, positive cultures from the injected sites were obtained as late as 8 weeks after this injection. If not treated, systemic side effects, including fever, malaise, myalgia, and abnormal liver function tests, persisted up to 2 months. In three cases toxicity was so severe that isoniazid was given, which resulted in prompt improvement. A severe hypersensitivity reaction occurred in a patient who was treated for the third time, 8 months after the second series of injections.—Natl Cancer Inst Monogr 39: 225–228, 1973.

BCG HAS long been used as a vaccine to prevent tuberculosis (*1*). Depending on the strain, the route of administration, and the dose of BCG, a conversion to a positive intermediate strength tuberculin test has occurred in 80 to nearly 100% of BCG-vaccinated individuals (*1*). Side effects from BCG vaccination, only rarely severe, consisted of indolent ulceration at the vaccination site, fever, lymphadenopathy, and hypersensitivity reactions (*1-4*). Deaths caused by BCG vaccination are extremely rare (<1 death in a million) and occur under circumstances that suggest immunological deficiency (*1*).

Apart from specifically protecting against tuberculosis, BCG can increase resistance to unrelated bacterial and viral infections (*5, 6*) as well as allografts (*7*) and can stimulate phagocytic activity and antibody formation (*8, 9*). More than 13 years ago, Old et al. and Biozzi and his co-workers reported that BCG delayed the appearance and ameliorated the course of experimental tumors in rodents (*10–13*).

Mathé et al. (*14*) first observed that percutaneous BCG vaccination of patients with acute lymphoblastic leukemia in remission induced by chemotherapy prolonged that remission (*14*).

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Morton et al. (*15*) reported that injection of BCG into cutaneous metastases of malignant melanoma caused regression of the injected nodules and occasionally of noninjected lesions.

We have conducted a similar study. Table 1 shows the extent of disease in our patients, all of whom were ambulatory at the onset of therapy. In addition to cutaneous metastases, 21 patients had lymph node metastases and 13 had metastases of lungs, bones, liver, or brain. The number of skin lesions was < 25 in 22 patients, 26–100 in 5 patients, and > 100 in 2 patients. We used BCG from Eli Lilly and Company, Indianapolis, Indiana, and the Research Foundation, Chicago, Illinois. The number of injected lesions ranged from 1 to 39, and the total number of viable injected organisms from 4×10^6 to 15×10^7 (based on the statement of the supplier). Individual lesions were injected with 5×10^5 to 15×10^7 organisms.

TABLE 1.—Extent of disease

Site	Number of patients
Skin, total No. of nodules	29
1–25 nodules	22
26–100 nodules	5
>100 nodules	2
Lymph nodes	21
Viscera	13

Table 2 summarizes the results of skin tests with 2,4-dinitrochlorobenzene (DNCB) and tuberculin.[3] Twenty-four patients had a positive DNCB test, and thirteen had a positive tuberculin test before therapy. Within 2 months after treatment with BCG, conversion to a positive tuberculin test occurred in 10 of 13 initially tuberculin-negative patients; these 10 were all DNCB positive. Of the 3 patients who did not convert, 1 was DNCB negative and 2 were DNCB positive.

TABLE 2.—Immune reactions of patients with malignant melanoma

Test	Number of patients	
	Positive	Tested
DNCB	24	29
Tuberculin		
Before BCG	13	29
After BCG	19	23

Side effects are shown in table 3. Most patients had fever, which lasted at least 2–3 days. Some patients, particularly those treated with larger doses, developed recurrent fever after 3–4 weeks, usually associated with an influenza-like syndrome consisting of nausea, vomiting, malaise, myalgia, and occasionally arthralgia. This syndrome was seen in 12 patients. Two were treated with isoniazid, resulting in rapid resolution. In untreated patients, these symptoms subsided with 8–9 weeks. Abnormalities of liver function tests, including glutamic oxaloacetic transaminase, lactic dehydrogenase, alkaline phosphatase, 5′-nucleotidase, and bilirubin, were observed in 14 patients, most of whom also had the influenza-like syndrome. Again, these abnormalities disappeared with the other side effects (unless the patient had progressive hepatic metastases).

TABLE 3.—Side effects of BCG

Type	Number of patients
Fever	26
Flu-like syndrome	12
Hepatic dysfunction	14
Hypersensitivity	1

BCG cultures were performed by Dr. Richard Costello, at Rockefeller University. Bacteremia existed only up to 5 minutes after injection. Even at the height of the flu-like syndrome, blood cultures were negative. However, material from draining lesions was positive for BCG as late as 8 weeks after injection.

One patient developed a severe hypersensitiv-

[3] DNCB was purchased from K & K Laboratories, Inc., Plainview, N.Y. Testing was performed according to the method of Brown et al. (*16*). Tuberculin was obtained from the National Communicable Disease Center, U.S. Public Health Service [purified protein derivative-ammonium sulfate precipitate: 0.0001 mg/0.1 ml (5 TU)] and from Parke, Davis and Company, Detroit, Mich. [Purified protein derivative-second strength: 0.005 mg/0.1 ml (250 TU)]. Patients failing to respond to 5 TU were tested with 250 TU. Only those patients who were nonreactive to 250 TU were considered tuberculin negative.

ity reaction. She had been treated with BCG twice before, 8 months and 12 months prior to the latest course of therapy. Within 6 hours after the injection of 54×10^6 organisms, she developed erythema not only around the nodules just injected but also around some nodules that were injected 8 months before. After 12–18 hours, the patient developed spiking fever and became restless, hypotensive, and oliguric. Acrocyanosis of fingers and toes occurred. The patient was treated with antihistamines, pressor amines, adrenal corticosteroids, and isoniazid. All abnormalities resolved within several days, except gangrene of the fingertips, which healed over a period of several months.

Table 4 summarizes the effects on the tumor. Twenty-five patients could be evaluated. Of the other 4 patients, 1 was lost to follow-up and 3 were treated with isoniazid 2–3 days after BCG had been given. Twenty-one patients developed an inflammatory reaction at the site of the BCG injection, followed by complete tumor regression in 10 cases and partial regression in 5. Cutaneous nodules not injected regressed simultaneously in only 2 patients.

TABLE 4.—Effects of BCG on malignant melanoma (intratumoral injection)

Number of evaluated patients	25
Inflammation of injected nodules	21
Complete regression of injected nodules	10
Partial regression of injected nodules	5
Regression of noninjected nodules	2

One of these 2 patients, a 56-year-old woman, presented with widespread recurrent melanoma of the skin of the left breast and back. On 12/10/70, 13 of the > 50 cutaneous nodules were injected with a total dose of 15×10^7 organisms. Erythema, induration, and ulceration of the injected lesions occurred 1 week later. After 1 month, the inflammatory reaction spread to the noninjected lesions. Four months after BCG injection, melanoma nodules were no longer demonstrable, and biopsy revealed only chronic granulomatous changes. The patient continues to be free of disease, 22 months after treatment with BCG. Although this patient's DNCB test was negative, she reacted to tuberculin (5 TU) before treatment with BCG.

The other patient, a 43-year-old woman, had 13 cutaneous metastases of the right thigh. Five were injected with BCG and regressed. Three of the eight noninjected lesions also regressed. Five months later, the remaining nodules were also injected and regressed. Since then, the patient has developed 3 new cutaneous nodules, 3–4 mm in diameter, which are growing very slowly and have not yet been injected with BCG.

Immunologic reactivity and therapeutic effect are correlated in table 5. Both the DNCB test and the tuberculin test were negative in only 1 patient; she did not respond to treatment with BCG. In all other patients, the DNCB test and/or tuberculin test were positive. Tumors regressed in patients of each of these groups. The first of the 2 patients who experienced regression of noninjected lesions (*see* above) was the one patient with a negative DNCB test. Of the 5 patients with initially negative tuberculin tests whose tumors regressed, 4 showed conversion of the skin test.

TABLE 5.—Correlation between immunologic reactivity and result of treatment with BCG

Immunological reactivity	Number of patients	
	Tumor regression	Evaluable
DNCB+, tuberculin+	4	7
DNCB+, tuberculin−	5	13
DNCB−, tuberculin+	1	4
DNCB−, tuberculin−	0	1

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Immunologic Approaches to Various Types of Cancer With the Use of BCG and Purified Protein Derivatives[1, 2]

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SUMMARY—Several approaches to BCG immunotherapy of skin cancer and related malignancies are presented. There were differences in responses of malignant neoplasms to BCG in immunocompetent as compared to immunoincompetent patients. Induction of hypersensitivity to BCG by the intradermal route and later by oral administration was used as a preliminary procedure for subsequent immunotherapeutic challenge with BCG or purified protein derivative (PPD). Moreover, we found that BCG not only induces a specific response to PPD but also indirectly establishes competency to react to other antigens.—Natl Cancer Inst Monogr 39: 229–239, 1973.

CLINICAL STUDIES

Initial studies on BCG[7] and purified protein derivative[7] (PPD) were carried out on skin cancers. Skin cancers usually occur in patients who have retained a substantial degree of immunocompetence. Furthermore, they lend themselves well for evaluation of clinical results with minimal and readily controlled side effects (*1–5*). Intralesional administration of BCG was initiated by infiltrating approximately 5×10^5 organisms (Connaught strain) in 0.5 ml of suspending fluid into a squamous cell carcinoma in a patient with arsenical dermatitis and a long history of multiple and metastatic epidermal cancers (figs. 1, 2). The patient had shown an intense response (4 +) to intralesional PPD (intermediate strength). A local reaction including edema, erythema, and vesiculation developed at the tumor site within 3 hours, while

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[7] BCG and PPD were obtained through collaborative studies with Dr. Silvio Landi, Head, Tuberculosis Department, Connaught Medical Research Laboratories, University of Toronto, Willowdale, Ontario, Canada.

normal skin (control) showed slight edema, consistent with the edema present after the administration of an equivalent amount of nonspecific protein. At the same time, the patient developed an elevated temperature, which rose to 103° F, chills, nausea, weakness, generalized joint pains, and swelling of the wrists and the joints of the fingers of the left hand. The systemic reaction responded partially to salicylates. The arthropathy persisted for 24 hours and gradually disappeared over the next 2 days. Systemic delayed-sensitivity responses, one of the complications of BCG therapy, are related to the degree of prior tuberculin sensitivity in the patient. While manageable by conservative measures, side effects may be anticipated and the patient must be observed closely during a 48-hour period following BCG inoculation (*see* "Discussion").

The intensity of the local reactions at the injection sites increased up to 48 hours after administration of BCG and subsided gradually over the next 3 weeks. The reactions were far more intense in the tumor than in normal tissues. Areas free of clinically detectable tumor were excised 2 months later and were grossly as well as microscopically free of tumor. Parallel studies with analogous results were carried out in patients with multiple basal cell carcinomas and premalignant keratoses.

Three patients with widespread premalignant and malignant epidermal tumors who had not responded adequately to other treatment modalities were treated with topical PPD. Topical PPD (100,000 U/g in petrolatum) almost completely cleared up the lesions in 2 patients, one with multiple superficial basal cell carcinomas and the other with squamous cell carcinomas. Another patient who showed less marked responses received oral BCG, followed by increased responsiveness to PPD. The effectiveness of intralesional and topical PPD for tumor resolution was directly related to PPD skin test responsiveness. However, the relatively slow antitumor activity of the PPD reaction was markedly increased by the addition of topical 5% 5-fluorouracil.

Six patients with mycosis fungoides were also treated with topical PPD. Three received BCG by intradermal (id) injection before or during topical or intralesional PPD administration. Three of those who initially reacted to PPD skin tests responded by complete clearing of their lesions after PPD administration. One patient was at the eczematoid stage (figs. 3, 4); a second was at the erythrodermic exfoliative stage, involving virtually the entire body surface; a third was at the ulcerative nodular stage and received intralesional PPD (figs. 5, 6). Complete disappearance of gross and microscopic signs of disease at sites following treatment lasted for ≥ 2 years. Administration of PPD involved: 1) twice daily topical administration of 10,000–100,000 U of PPD/g of petrolatum or 2) intralesional administration of 2.5–1,000 U of PPD at intervals of 2 or 3 days ranging from 1 dose (2.5 U) to 36 consecutive doses. Of the 3 other patients with mycosis fungoides who received id BCG (5×10^5 organisms in 0.5 ml) at intervals of 2–4 weeks for 3 months, 2 showed increased responses to PPD. Both were at the plaque stage of the disease and showed limited responses to PPD. The third patient, who had total erythroderma, did not show an increase in PPD reactivity and failed to respond therapeutically. BCG and PPD administration was discontinued, and treatment with triacetyl-6-azauridine (TAU) (Azaribine [8]) was initiated (50–250 mg/kg) (figs. 7–9).

Findings similar to those made in skin cancer and mycosis fungoides were obtained in patients with adenocarcinoma of the breast (7 patients), with reticulum cell sarcoma (2 patients), lymphangiosarcoma (postmastectomy Stewart-Treves syndrome—1 patient), Kaposi's hemorrhagic sarcoma (2 patients), and malignant melanoma (3 patients). One patient with reticulum cell sarcoma responded to challenge with intralesional PPD. Only 5 of 17 lesions regressed completely after intralesional PPD (2.5–100 U/injection for 3–20 consecutive injections at intervals of 2 or 3 days). Consequently, the patient was placed on oral BCG (50 mg/dose) which was administered at intervals of 1–2 weeks for 6 months. During that time, the responsiveness to PPD as well as to a number of other microbial antigens increased markedly. Repetition of challenge with

[8] Azaribine was provided through the cooperation of Mr. Charles A. Hoppes, Clinical Associate, Calbiochem, San Diego, Calif.

PPD or BCG intralesionally resulted in regression of injected tumors as well as lesions at distant, unchallenged sites (figs. 3, 10, 11).

The patient with lymphangiosarcoma initially was mildly PPD positive. She was subsequently given intralesional injections of BCG and challenged with PPD. Both injected lesions as well as noninjected lesions at distant sites underwent delayed-hypersensitivity reactions and regressed. Because of the widespread involvement of the lesion, the patient was subsequently given an intralesional injection of vinblastine (a single injection of 1 μg in 0.1 ml into a lesion approximately 1 cc in volume). The injected lesion underwent necrosis within 24 hours but continued to show residual malignant cells at its margin. Analogous lesions were subsequently injected with PPD and vinblastine (0.1 μg in 0.1 ml of suspending fluids) as well as with PPD (2.5 U in 0.1 ml) and appropriate control injections.

The sites at which PPD was administered showed complete regression, while variable tumor cells were found at the other sites. The patient was subsequently treated with systemic vinblastine at relatively low doses (0.1 mg/kg) at intervals of approximately 7–14 days, depending on the depression of the peripheral leukocyte count. Oral BCG was administered at those intervals during which the white blood cell count had returned to normal levels following parenteral administration of the drug. Over a 5-month period, the disease, which had been rapidly progressive at the time of the patient's admission, showed no further progression. Five of 6 biopsies taken at random were negative for residual tumor that had been taken from previous biopsy-confirmed tumor sites. Abnormal liver function tests (elevated alkaline phosphatase, lactic dehydrogenase, and serum glutamic oxaloacetic transaminase) returned to normal. A mild anemia which had been present reverted to normal hemoglobin levels.

Two patients with Kaposi's hemorrhagic sarcoma showed local as well as distant regressions after administration of BCG and challenge with PPD. One of the patients with Kaposi's sarcoma was virtually nonresponsive to our complete battery of skin test antigens before id administration of BCG. After 6 successive administrations of 5×10^5 BCG organisms, the patient showed a positive reaction to PPD (second strength) and became positive to mumps antigen, Varidase, and *Candida* extract. At the same time, intralesional challenge with PPD or BCG resulted in local as well as distant regression of lesions. Over a 3-year period, the lesions which had undergone regression failed to recur. No new lesions have developed.

Three patients with malignant melanoma were also studied in this group. All were nonresponsive to skin test antigens before administration of BCG. BCG was administered initially intralesionally to 2 of these patients and orally to the third patient. Intralesional administration resulted in conversion to a positive PPD response as well as conversion to other skin antigens (table 1). One of the 2 patients who had been inoculated intralesionally with BCG showed local as well as distant regressions of cutaneous melanoma during continued intralesional administrations of BCG and/or PPD. The patient, who

TABLE 1.—Effect of BCG immunization on increase in PPD skin test response and positive conversion of other skin test antigens

Tumor	Total No. patients treated	Number responding (BCG)	Number showing increased PPD response	Number showing positive conversion to other skin test antigens*
Epithelial skin cancers	7	5	3	4
Mycosis fungoides	6	3	3	3
Carcinoma of breast	7	7	2	4
Other tumors	8	8	3	4
Total	28	23	11	15

* Skin test antigen battery included: PPD (first and second strength), mumps antigen, *Candida* extract, trichophytin, histoplasmin, Schick antigen (*Corynebacterium diphtheriae* toxin), and Varidase. The conversion from negative to positive skin test response after inoculation with BCG is statistically highly significant even in this small sample ($P > 0.001$ as determined by χ^2 analysis of a 2×2 contingency table for association).

was sensitized to BCG by oral administration initially once a week and subsequently 2 or 3 times a week, responded to intralesional administrations of BCG or PPD by regressions of injected tumors and of tumors at distant, unchallenged sites. Although 50% of the lesions in this patient regressed for periods of 12–18 months, the lesions no longer continued to regress, but subsequently expanded. New lesions formed at other sites; lesions, which had previously regressed, began recurring, despite continued BCG administrations and PPD challenge. Our experience with BCG and related or concurrent therapy is summarized in tables 1 and 2.

DISCUSSION

Our studies have shown that the oral route of administration of BCG is highly effective in inducing responsiveness to PPD in patients who were previously anergic, in agreement with the findings of Thomas and of Falk (personal communications). Furthermore, patients who responded weakly to PPD initially demonstrated marked reactions after BCG administration. In addition, most patients receiving BCG who had been nonresponsive to a battery of skin antigens and nonresponsive to dinitrochlorobenzene (DNCB) showed onset of responsiveness or increased responsiveness, respectively, after administration of BCG.

Oral administration of BCG has the advantage of not producing cutaneous ulcers or vesiculation. The side effects of oral BCG are similar to those of id BCG, although the dose given orally is considerably larger. Side effects include a flu-like syndrome with chills, elevated temperatures up to 103–104° F, arthralgias, weakness, nausea, and rarely vomiting and diarrhea. The onset of these side effects is usually within 24 hours after oral administration and may occur as early as 3 hours or as late as 48 hours after the agent is administered. As a rule, mild analgesics, antipyretics, and antispasmodics are sufficient for controlling these side effects. Anaphylactic reactions and clinically significant BCG infections have not been found in this group of 28 patients.

To obtain maximum benefits from BCG, it must be given in relatively large amounts at frequent intervals for a protracted period. While a single administration may induce hypersensitivity to PPD, it will not lead to activation of the reticuloendothelial system and a general increase in cell-mediated immune responsiveness to unrelated microbial antigens, such as *Candida* extract, mumps antigen, or PPD. On the other hand, continued administration of BCG results in successive increases in PPD responsiveness as

TABLE 2.—Response of patients with diverse tumors to BCG or PPD immunotherapy

Tumor	Total No. patients treated	Regressions following treatment					
		Number responding (BCG)	At injected lesions (BCG)	Distant sites (BCG)	Number responding (PPD)	At injected lesions (PPD)	Distant sites (PPD)
Epithelial skin cancers	7	5	5	1	3	3	0
Mycosis fungoides	6	3	3	2	3	3	3
Carcinoma of breast	7	7	5	1	7	2	1
Reticulum cell sarcoma	2	2	1	0	1	1	—
Lymphangiosarcoma	1	1	1	1	—	—	—
Kaposi's sarcoma	2	2	1	1	1	1	0
Melanoma	3	3	2	1	2	1	0
Total	28	23	18	7	17	11	4
Frequency of responders		0.82*	0.78†	0.30†	0.61*	0.64†	0.24†

* Frequency of responders compared to total number of patients studied.

† Frequency of patients responding at the injected lesion or distant sites compared to the total number of responders in each of the BCG and PPD categories. At present, the number of patients studied is too small to allow definitive conclusions on the effectiveness of either form of therapy or regression of distant lesions. The differences in responders are not statistically significant ($P > 0.05$ by the standard error of the difference between 2 proportions) when the 2 modes of therapy are compared. It must also be remembered that the PPD group responded with 4+ skin tests, whereas the BCG group had to be immunized.

well as reactivity to other cell-mediated antigens.

Correlating the course and prognosis of malignant disease with the patient's immune status is still a difficult job. Although a number of investigations have reported that the presence of immunocompetence increases the outlook for a favorable prognosis, a quantitative, statistically reliable population sample size has not yet been studied. For example, we have observed that a considerable number of patients with progressive malignant diseases were immunocompetent as determined by presently available methods. The clinical detection of impaired immunocompetence in patients with malignant disease appears to indicate a bad prognosis and an unfavorable course. Since patients with apparently normal immunocompetence develop malignant disease, which progresses despite their apparently adequate immune status, apparently other methods besides skin testing should be investigated to detect the critical diagnostic features of the complex cellular immune response components which may confer resistance to cancer.

Although the skin test challenge response to cutaneously administered, sensitizing antigens appears to predict the patient's prognosis, this relationship is not sufficiently documented for reliably predicting a patient's outlook with certainty. In vitro tests, such as lymphocyte stimulation, macrophage-migration inhibition, or target cell destruction by the patient's donor lymphocytes, have not yet been studied extensively enough to replace skin testing. Humoral antibody determinations also have not shown a direct relationship to prognosis in cancer. By pointing out the paucity of information, we by no means want to discourage such studies, but in fact hope to encourage an intensified effort at gathering data. The presence of blocking or enhancing antibodies, as described by Bansal and Sjögren (*6, 7*), and other serum-blocking factors justify considerable further investigation into the correlation of the course of malignant disease, with quantitative determinations of these factors.

Animal systems have provided models for the study of BCG and other factors playing a role in cell-mediated immunity (*8, 9*). These animal models have permitted us to evaluate individual factors systematically pertaining to the immune system which interact in the resistance to malignant disease. Although considerable insight into these relationships has been gained from studies in the guinea pig (*8, 9*), a great deal of additional work is required to elucidate those factors of proven significance for malignant disease in man.

Our studies as reported here are still at an early stage of exploration. The time elapsed since studies were initiated has been too brief and too few patients have been observed to permit definitive conclusions. No doubt, many subtle details pertaining to immunologic manipulation, with particular reference to BCG and PPD, have yet to be determined. These include: differences between various strains of BCG and their relationship to different types of malignant disease; dose, frequency, and route of administration; and schedule of treatments. Similar considerations pertain to the various kinds of antigens used both for testing and for investigative therapy, such as PPD, *Candida* extract, Varidase, mumps, and others. These considerations also pertain to synthetic organic chemical agents, such as DNCB, and any one of the various agents by which immune responses may be induced or potentiated.

At the present stage, several other microbial agents have been reported to act as immunopotentiating agents with apparently favorable effects on the course of malignant disease. *Corynebacterium parvum* and *C. granulosum* as reported by Israel (*10*) and other agents which have been less intensively studied require considerable additional attention. Our studies (*5*) with multiple antigens inducing therapeutic effects when used concurrently (while individual agents used by themselves are ineffective) suggest that combinations of appropriate antigens act synergistically in immunopotentiation.

Although the studies recorded here are not conclusive in terms of therapeutic effects, they clearly point out that, in advanced states of malignant disease, our present techniques of enhancing immunocompetence are still not adequate to achieve significant therapeutic effects. The data presented previously (*5*), describing the effect of combining immunotherapeutic approaches with chemotherapy and/or with ionizing radiation therapy (*11*), suggest that combining several modalities may improve thera-

peutic efficiency when compared to single modalities. Thus the data obtained with chemotherapeutic agents which do not significantly suppress immunity, such as TBU or bleomycin, need to be augmented. Similar considerations pertain to the use of cytotoxic agents which should be used on a restrained basis to permit rebound of immunocompetence between successive courses of chemotherapy. Examples of this approach are referred to above (*12*) using bleomycin, Cytoxan, or vincristine in combination with immunologic approaches. Similar considerations pertain to the use of the high-dose methotrexate-citrovorum rescue procedure described as a pulse-therapeutic approach by Djerassi et al. (*13*).

From the available information at present, we conclude that protocols must be designed to gather data in an adequate number of control and treated lesions for statistical analysis and, where appropriate, double-blind experiments must be done. Our data obtained so far have been collected for the purpose of pilot, descriptive investigations to explore several modalities of treatment for patients with both contained and widespread disease. Our primary goals for future studies are quantitative assessment of immunologic reactions, dosage, route of administration, and administration schedule and correlation of these parameters with clinical status of the patient.

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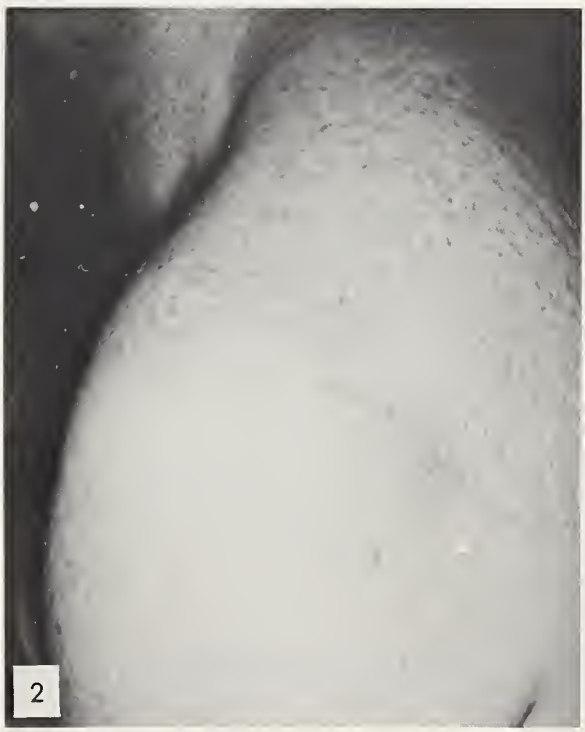
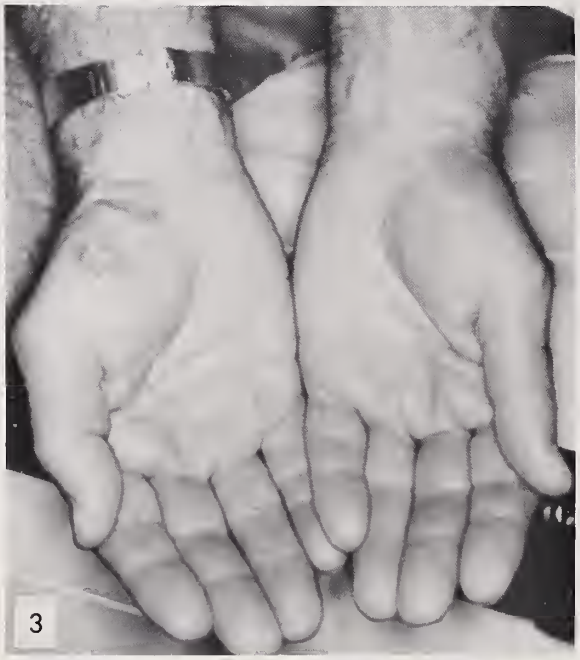


FIGURE 1.—Patient with arsenical dermatitis and multiple squamous cell carcinomas. Lesion is shown following intralesional injection of BCG. Intensity of reaction has started to subside.

FIGURE 2.—Same area as shown in figure 1, 6 months later following disappearance of lesion. Excision biopsy of area was grossly and microscopically negative. No recurrences were noted for 2 years.



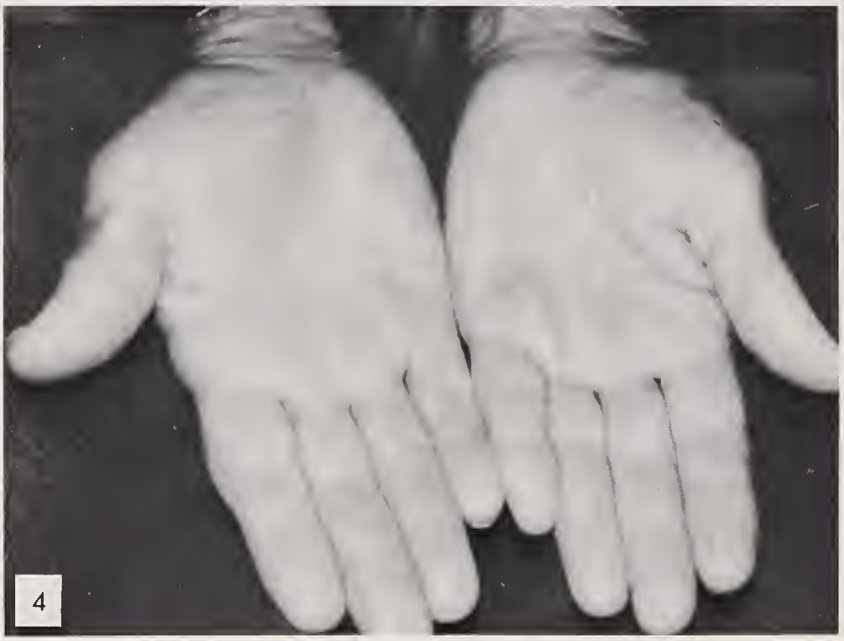


FIGURE 3.—Patient with mycosis fungoides at eczematoid stage.

FIGURE 4.—Same patient as shown in figure 3 after immunotherapy with topical PPD.

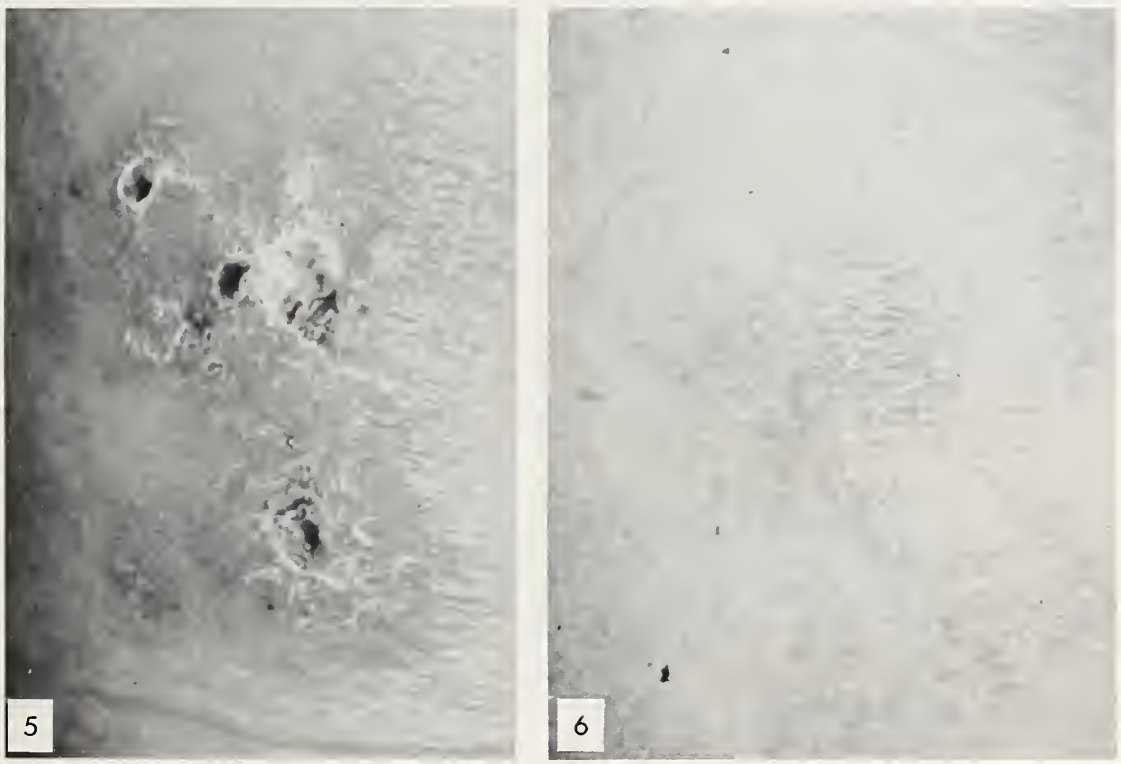
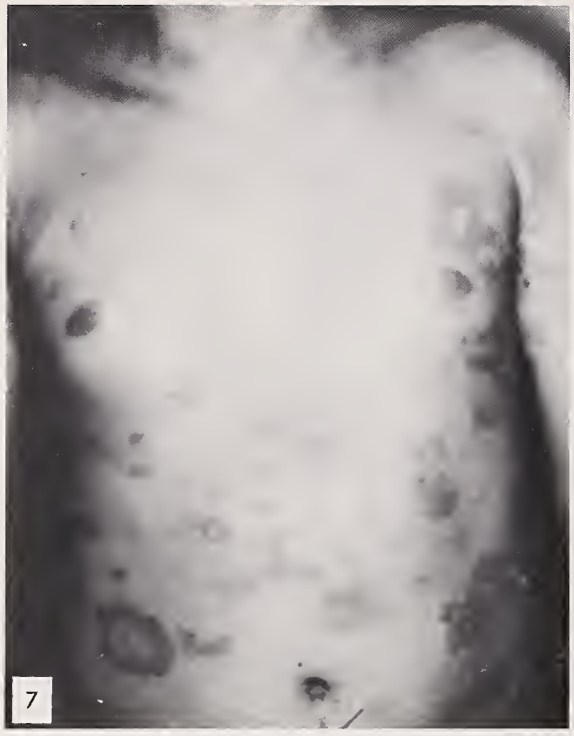


FIGURE 5.—Patient with mycosis fungoides—ulcerative nodular stage during intralesional injections with PPD.

FIGURE 6.—Same area showing complete regression. Multiple biopsies indicated that the site remained negative for $>$ 3 years.



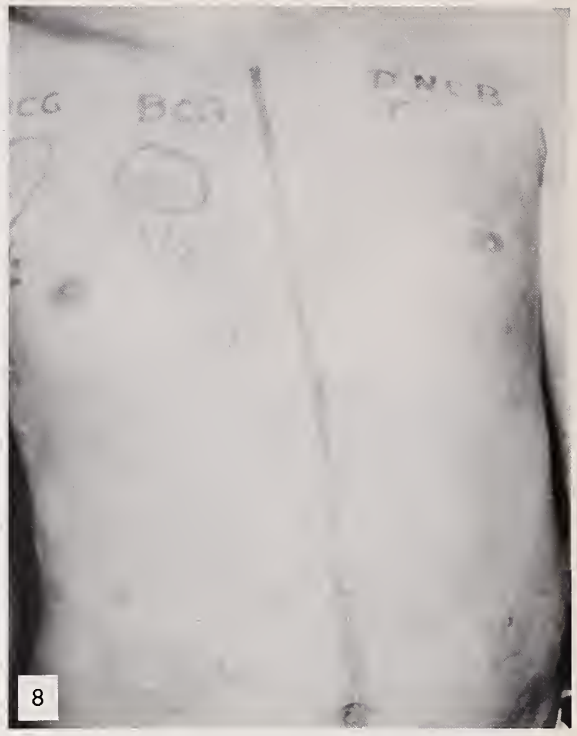


FIGURE 7.—Patient with mycosis fungoides at plaque stage after courses of separate modalities of therapy: 1) immunotherapy and 2) chemotherapy (systemic TAU). Response and progression of disease were only marginal.

FIGURE 8.—After treatment with combination of chemotherapy (TAU) and immunotherapy (BCG, PPD, and DNCB), lesions responded with clearing.

FIGURE 9.—Same patient as in figures 7 and 8, two years after, following regimen of combined chemotherapy and immunotherapy (described in fig. 8 and text). Lesions have cleared and disease remains under control.

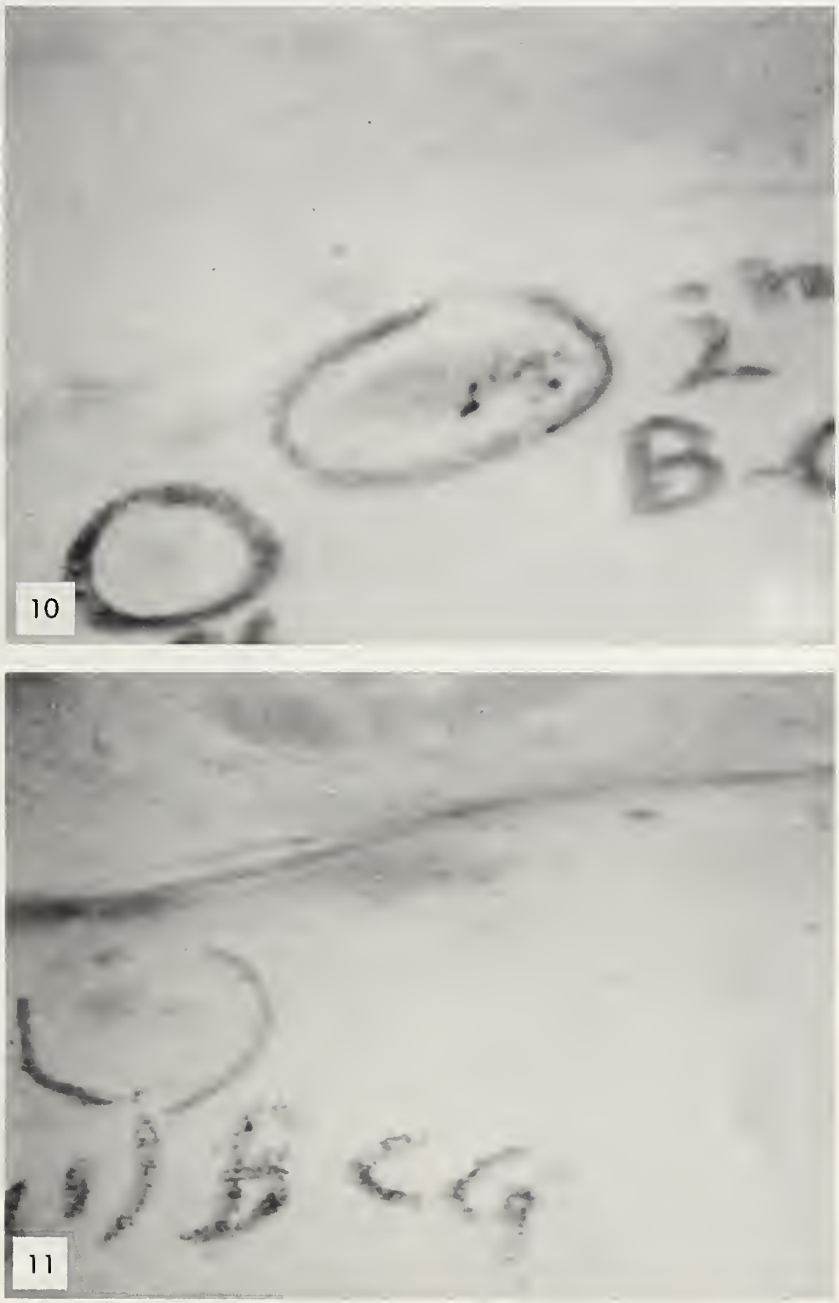


FIGURE 10.—Site of metastatic adenocarcinoma of breast, before immunotherapy. Patient had failed to respond to standard forms of therapy.

FIGURE 11.—Same lesion 3 weeks after intralesional injection of BCG. Site is free of gross and microscopic evidence of malignant cells.

DISCUSSION

C. G. Zubrod: As a chemotherapist, I have enjoyed hearing about the problems faced by the immunotherapists. As far as chemotherapy goes, it's reminiscent of the early 1950's when drugs were being found that could make a tumor go away. And these were qualitative observations. Gradually everybody began to realize that we were faced—in trying to correlate animal disease with human disease and in trying to study the human disease quantitatively—with enormous numbers of variables: different drugs, different purity of drugs, different animal models, different routes of administration, different amounts of drug that would be administered, many different human diseases, etc.

But we did work these problems out. I'm not at all proud of the result because it took us over 10 years in a learning process to realize that, if we did things differently in an animal system than we did in man, we couldn't make any correlations.

Once we began to standardize quantitatively all the variables in the animals and all the variables in man in a highly controlled comparative way, the correlations, along about 1966 and 1967, suddenly became apparent, and there was indeed excellent predictability of the animal studies for the clinical situation.

I hope that the immunotherapists, equipped with several substances which seem to cause tumor regression, will not go through a similar learning period, but will take advantage of some of the mistakes we chemotherapists have made.

First I would recommend, therefore, that out of these meetings would grow some operating group who would attempt some forms of standardization—not in order to suppress imaginative research but to foster the kind of quantitative observations in animals and in man so that we will be able to come up with more than the kind of qualitative conclusions that we have been able to reach at this meeting today.

Secondly I would recommend that the clinician be cautious in setting up studies in man. The physician faces many ethical dilemmas in trying to apply these results to clinical trial. These materials apparently can cause, under certain unknown circumstances in animals, a regression, no effect, or a sudden acceleration of tumor growth as a result of the immunologic manipulation. We ought to know much more about those conditions in the animal in which regression will occur and those in which tumor growth will be accelerated. Until this is clearer than it appears to be now, one would wonder about the wisdom of very widespread trials of immunotherapy at this moment.

Regarding other ethical dilemmas, Dr. Rapp stated yesterday that BCG, or the other materials, must come in contact with the cell. Most of the evidence which I have heard seemed to show that this was indeed a local form of treatment, though there are some suggestions that there is some systemic effect.

There are certainly systemic effects with regard to immunoprophylaxis. Whether systemic effects exist with regard to immunotherapy seems questionable to me at the moment.

Now, if a disease is curable by local therapy, one has to take into account—as Dr. Klein pointed out this morning in the preferred treatment of squamous cell cancer—that there are very good means, radiation or surgery, of removing local disease. So we have, therefore, the problem of choosing in an ethically justifiable way which patients on whom we can carry out these sorts of studies.

The dilemma is heightened by the fact, as a number of other investigators have mentioned here, that BCG is effective against relatively small numbers of tumor cells —10 million is one figure I remember from the work reported yesterday on rats—and yet these tumors would be the types which should be surgically easily curable. The trials are justified only in patients with very advanced disease. And yet, do the experimental data show that these materials will be active in very advanced disease?

These are some of the ethical dilemmas that we must face in designing clinical trials. They can be overcome, but I would urge you, as a result of these meetings, to try to get together—first to try to quantitate your variables so quantitative studies in animals and men can be run and secondly to get some of the clinicians to think about how to design meaningful trials taking into account these ethical cautions.

Klein: Are there any comments on Dr. Zubrod's remarks?

D. B. Windhorst: As I mentioned this morning, as a clinician I think most of us still recognize that we have to go from the anecdotal observation to the controlled, randomized, clinical study in this area. I am involved in some of these attempts.

Specifically, I am involved in a joint effort with Dr. Klein to devise a properly controlled clinical study for evaluating topical therapy in actinokeratoses and superficial nodular basal-cell carcinoma. So in that area we're faced with very difficult problems of defining a disease to be treated and the patients to be taken into the randomized process and then of measuring the response. These are problems to which Dr. Zubrod alluded when he referred to the ways of examining clinical trials.

The problems in these various areas are very real and in this general area of immunotherapy probably are highly analogous to those of chemotherapy.

Another area in which I am involved is an attempt, in a more general way, to register the work currently being done by various people with immunotherapy of any kind. In fact, Dr. Terry has scheduled a meeting of people interested in this kind of work in man, so that we can begin to accumulate the data, regardless of the type of protocol involved. We can examine these various studies and perhaps put some of the smaller studies together to get additional information.

I do want to emphasize that there are some differences in doing this in immunotherapy versus chemotherapy. We cannot take the models that chemotherapists have given us and immediately apply them to immunotherapy,

because immunotherapy has some additional variables which have become very evident to me as I've tried to work out some of these designs of registry forms and the like. The variable that is most unique in immunotherapy is that we are dealing with a definable host system—not so very definable, but to some extent the host status with regard to his immune capacity in relation to 1) the tumor and 2) whatever adjunctive immunotherapy other than tumor cells that might be given.

These must be measured before, during, and after treatment before we can know what is happening in immunotherapy. And if they are going to be measured—and they have to be—we must record them in this registry about which I'm talking.

So this is by way of defending what I was saying earlier this morning and by way of saying we are after this goal, but I think we've got a much more complex problem. It's going to perhaps take much longer than the chemotherapeutic trials to arrive at competent data, which we hope to achieve.

Klein: Perhaps some of the things you mentioned are included and accounted for in the chemotherapy programs because the immunologic backup of the animal or the patient might have some very important bearing on their responses to chemotherapy; conversely, the effect of chemotherapy on the immunologic capabilities is a facet that ought to be of direct concern to those who are primarily working with chemotherapy.

M. Chase: We heard this morning some excellent really raw data, and I have been a little appalled by some of the strong reactions to the BCG vaccine. I would like to make a couple of comments about the repeated administration of BCG.

Purified protein derivative (PPD) is not a sufficient material to pilot what is happening to your patient. The organism also has another heat-labile protein that is destroyed in the making of PPD, and the sensitivity that arises to this protein does not parallel that to the heat-stable constituents. Also, we have seen antigens of the cell wall. If we really want to protect the patient from repeated administration of BCG and to pilot it, perhaps a phenol-killed BCG preparation in highly dilute suspension, in phosphate-buffered saline containing Tween at 5 μg/ml, could be used as an intradermal challenge. Thus we will measure all the antigens that are functioning and how the patient is responding to these antigens.

M. Lasker: Is it only with BCG that tumor growth is sometimes enhanced or also with PPD and other immunogenic agents?

Klein: From our fairly lengthy experience, we have not seen enhancement with other agents, but the principles I would assume would be the same and enhancement might occur.

At the same time there are no convincing data, certainly no statistically significant data, that BCG has indeed increased the progression of tumor development.

Melanoma is a very peculiar disease, characterized by the fact that the patient may go on for 20 years with residual melanoma and within 2 months progressive melanoma will develop and the patient will die. And those patients who are put on the immunotherapy schedule are almost invariably those who are in very bad condition. So you are certainly weighting the chances in favor of having spontaneous exacerbations.

P. Alexander: How do the speakers who have just reported on the effects of treating melanoma with intralesional injection of BCG justify continuing this procedure in the light of the results so far obtained? My impression is that the treatment with local BCG is much more traumatic (and even has some systemic toxicity) than surgery or one of the many other ways available for treating local lesions. The fact that in 1 case in 10 or 20 an effect is seen in a noninjected lesion does not seem to justify use of this treatment, since regional perfusion with cytotoxic chemicals has been shown to affect melanoma lesions at a much higher incidence than 1 in 10 or 1 in 20 as claimed for the indirect effect of BCG. Moreover, by no means is it evident that this is necessarily a systemic effect in those rather rare cases in which a lesion not injected with BCG regressed after injection of another lesion. Often the responding lesion has been quite close to the injected lesion, and conceivably some of these rare instances in which noninjected lesions regress could be attributed to BCG organisms draining into the tumor.

Klein: Dr. Nathanson, doesn't regional perfusion with phenylalanine mustard last, on the average, around 3 weeks?

L. Nathanson: The average duration of response is much greater, about 40 weeks in our hands. But perfusion is technically cumbersome, is traumatic, and is now being done only in a few centers. It has been largely abandoned because the overall results do not appear to justify the effort and expense of the procedure.

But the patients, at least those with melanoma, who undergo regression of uninjected lesions, and in fact, occasionally of all clinically demonstrable lesions, provide us with a human model for possible immunologically mediated tumor regression. This human model, if studied, is likely to yield the most interesting possible information about mechanism, if the appropriate in vitro systems are set up in which to obtain that information.

Thus, quite apart from whatever clinical improvement one sees in these patients—and like Dr. Pinsky and Dr. Bornstein we have seen some very helpful clinical responses—the information which may be derived from these patients is almost unique and provides us with an opportunity which we can ill afford to pass up.

S. R. Rosenthal: I want to add in answer to Dr. Alexander that, when one injects the melanoma cells with BCG, one gets a high increase in titer of complement fixation or cytotoxicity to the melanoma antigen, which is directly related to the prognosis of the disease. So there is an added advantage of giving specific antigen and nonspecific antigen in cases of malignancy.

B. H. Waksman: I agree with Dr. Nathanson. You have a perfect guinea pig situation in the human subject. But the problem is that we know how to look for certain things—and the last several papers that we heard show one can do proper in vitro tests—but that the information gathered so far has not precisely defined what

it is we're doing that is favorable to the tumor. So maybe we should be looking for something more than we are looking for at the moment.

And I plea for being experimental about the in vitro tests as well. For example, in one of the papers, there was a comment that none of the parameters changed in in vitro reactivity to phytohemmaglutinin, etc.

Perhaps not only must we test for reactivity but also we need to have dose-response curves to see whether the level of reactivity of the lymphocyte is the same as that in the normal individual. Maybe we also need reasonable in vitro tests to see how macrophages are behaving in these patients. I'm sure we could devise such an in vitro test, or something like it.

C. M. Pinsky: I should say a word about Dr. Alexander's comment, which may also apply to Dr. Zubrod's ethical question.

In the choice of these patients, due consideration was given to their previous course and ultimate prognosis. Most patients, even those with few lesions, had had multiple metastases that continued to appear despite excision.

Then you may be justified in doing something that at least smacks of the possibility of a systemic effect (a combination of immunotherapy and perhaps immunoprophylaxis) . This is what needs to be defined better.

But I would be the first to agree that, if a primary lesion in a patient is diagnosed as malignant melanoma, the appropriate surgical procedures should be carried out.

Klein: We have two questions at issue here. 1) What is the practical management? 2) What can we learn about it?

With regard to practical management, obviously the patient ought to be treated as well as possible. With regard to learning, we can learn from human lesions, Dr. Alexander, as much as we can from animal lesions.

Alexander: I question that.

Klein: I know you do, but ultimately man will be your court of appeal. It has been the case in chemotherapy. It doesn't mean we shouldn't use animal models.

If Dr. Zubrod had stopped giving his patients anything while he was studying these animals, I don't think this would have been proper. Certainly the brilliant success that chemotherapy has had from running both experiments at the same time is something from which we should learn. We certainly cannot abandon man.

H. F. Seigler: We must stress one point. I've been a bit unimpressed with some chemotherapy and often the cooperative efforts have led to cooperative confusion.

All of us have seen patients who respond, which is interesting, but response becomes only really meaningful and applicable to us when we can say not that 50% responded, but predict which 50% are going to respond.

That leads toward the one thing absent in much of chemotherapy, very scantily reported at this meeting so far, and mainly going back to what Dr. Waksman mentioned yesterday: mechanisms.

There are very good means now for studying mechanisms in the immune response. Rather than trying to say, "Let's assume we are in the position now of cooperating and getting together and finding protocols," we ought to stress that everybody should return to their laboratories and look at mechanisms in their own protocol. Only when we can define those mechanisms can we apply these definitions to therapeutic maintenance, which can be done in humans.

N. Sher: Dr. Bornstein or Dr. Nathanson, does either of you have any data on the time course in the development of the serum-blocking factors to which you alluded in regard to your patients who did not do well with the first administration of BCG; in patients like these who are being monitored, should the frequent administration of BCG be stopped?

R. S. Bornstein: Our data are just now being rigorously analyzed. Apparently many of the patients who did not respond, or who perhaps had their disease accelerated, had the blocking factor present before treatment. These serum-blocking factors did not seem to develop in most of the patients after treatment. There are several patients in whom these factors seemed to develop early in the treatment, but in some of those patients we could not identify cytoxicity before treatment so that adequate measurements could not be done.

Nathanson: We have done a couple of very preliminary, serial studies which suggested again, as Dr. Bornstein has mentioned, that it is possible to convert patients from positive to negative blocking and to sustain them in a state of negative blocking. But the whole point of this exercise is that, if we don't further examine patients with the system, we'll never know whether these mechanisms are of use. In fact, more important than that, we will never know whether the animal models are analogous to the human models and therefore whether further animal work will shed light on the human situation.

J. E. Sokal: Dr. Nathanson, how much cross-reactivity exists between melanomas of different patients as measured by your cytotoxic and blocking assays?

Nathanson: The cytotoxicity assay seems to have good cross-reactivity, as evidenced by the fact that we use allogeneic cell lines. In a significant number of patients, we can obtain repeated biopsies, whereas in some patients we can't obtain any biopsies. Therefore, we can't run a totally autologous system, including target tumor cells and lymphocytes. So we believe that cross-reactivity in general is good and that cell lines can be used as the target system, so long as HL-A typing and other means are used to rule out histocompatibility problems between lymphocytes and target cells.

P. Salk: In any of those investigations in which noninjected cutaneous nodules regressed, were attempts made to identify the BCG organism in the regressing nodules?

Unidentified speaker: We have and we've been unable to find it.

Nathanson: We have never been able to culture BCG organisms out of distant sites from BCG injections.

Immunotherapy of Acute Myelogenous Leukemia in Man[1, 2, 3]

Ray Powles, *I.C.R.F. Oncology Unit, St. Bartholomew's Hospital, London, and Chester Beatty Research Institute, Institute of Cancer Research, Royal Marsden Hospital, Sutton-Surrey, England*

SUMMARY—Forty-six patients with acute myelogenous leukemia were included in a study of the effects of immunotherapy with BCG and irradiated allogeneic myeloblastic leukemia cells on remission duration and survival. After full remission was achieved with rubidomycin and cytosine treatment, the patients were randomly assigned to one of four maintenance groups: intermittent chemotherapy; chemotherapy plus immunotherapy; continuous, moderately intensive chemotherapy; and immunotherapy only. Median remission duration was 155 days for patients (31) receiving maintenance chemotherapy; only 24 had relapses. Median remission duration was 430 days for patients (15) scheduled for maintenance chemotherapy plus immunotherapy; 5 had relapses. Of the 15, 2 had so far received immunotherapy only: 1 had a relapse at 520 days; the other remained in full remission at 661 days. Survival data were analyzed 5 months later. Median survival for 14 intermittent chemotherapy patients was 45–85 weeks, with 7 still alive. Median survival for the chemotherapy plus immunotherapy group could not be determined because only 4 of 15 had died. Separate analysis of 9 patients receiving only immunotherapy indicated survival figures similar to those for patients receiving chemotherapy plus immunotherapy.—Natl Cancer Inst Monogr 39: 243–247, 1973.

WITH ONE notable exception (1), there is no evidence that immunologic procedures are useful for the treatment of cancer in man (*2, 3*). Therefore, the design of tumor immunotherapy programs should include every possible way to make them so. Five important factors guided our group to choose acute myeloblastic leukemia for a clinical study of immunotherapy. I will briefly discuss these factors and then present the clinical results of our study.

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] Supported by the Joseph Frazer Strong Trust, Medical Research Council, Leukaemia Research Fund, and Imperial Cancer Research Fund.

[3] A cooperative program at St. Bartholomew's Hospital I.C.R.F. Oncology Unit under the direction of Gordon Hamilton Fairley, Derek Crowther, and Colleagues; and the Leukaemia Unit, Royal Marsden Hospital and Chester Beatty Research Institute, under the direction of Peter Alexander, Humphrey Kay, and T. J. McElwain. We are indebted to Dr. P. E. Thompson Hancock for his guidance in this program.

Large Mass of Chemotherapeutically Sensitive Tumor

Data have repeatedly shown the importance of using tumor cells for immunotherapy of tumor-bearing animals (*4*). Currie et al. (*5*), studying malignant melanoma in man, found it critical that tumor cells be available in large quantities and that there be minimal residual disease before immunization. Acute leukemia in man meets that criterion: Many cells may be collected from the blood of leukemia patients before treatment; often after only short periods of chemotherapy, no detectable disease remains.

Tumor Antigenicity

We previously showed that stored leukemia cells from patients with acute leukemia stimulate in vitro DNA synthesis in autologous lymphocytes (*6*). This suggested the presence of material in the surface of the stored cells which behaves like an antigen. This stimulation of DNA synthesis, produced by both lymphoblastic and myeloblastic leukemia cells, was not the result of the storage procedure, because no stimulation occurred when stored remission bone marrow containing no leukemia cells was identically treated and tested (separate experiments).

Host Response to the Antigenic Tumor

In the same experiments, we demonstrated that human leukemia cells may be specifically immunogenic in man. We observed the change in the leukemia cell recognition by lymphocytes after the patients were immunized with a large dose (1×10^9) of their own stored irradiated leukemia cells. In most cases, the response was only transitory. It was usually specific for the leukemia cell antigen; but occasionally the mixed lymphocyte reaction, if severely depressed, was also enhanced by autoimmunization.

Cross-Antigenicity

Because of interference by transplantation antigens in the surface of leukemia cells, mixed cultures cannot be used to determine directly whether the antigen(s) in human acute leukemia is individually specific. However, by adding sera (taken from the patients on the same or previous occasions) to these reactions, we found factors which only sometimes inhibited leukemia cell recognition and were not patient specific (*7*). Thus the leukemia antigen may be common to all patients.

Normal Cell-Mediated Immune System in the Host

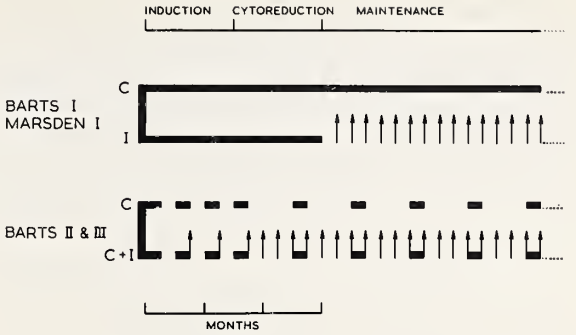
In unpublished experiments, we found that patients with acute lymphoblastic leukemia, even up to 2½ years after chemotherapy, have a defect of cell-mediated immunity that gives abnormally low lymphocyte reactivity in the mixed lymphocyte reaction. This is probably not the result of previous intensive chemotherapy, because patients "cured" of chorioepithelioma by similar chemotherapy had normal mixed lymphocyte reactions. Because it is not ethical in Britain to withhold treatment from patients with myeloblastic leukemia in remission, lymphocyte function in those patients cannot be determined. However, in patients receiving only immunotherapy—weekly subcutaneous (sc) BCG and allogeneic leukemia cells—the mixed lymphocyte reaction reverted to normal in myeloblastic but not in lymphoblastic leukemia.

These 5 factors indicated to us that the remission phase of myeloblastic (unlike lymphoblastic) leukemia was a good clinical situation for detecting possible therapeutic response to immunologic procedures. We also believed that immunization should consist of frequent, long-term injections of many irradiated leukemia cells.

In animal experiments, immunization with cells sharing a common tumor antigen increased therapeutic benefit. Fortunately, this is the only logistically feasible method of treating man as long as the leukemia antigen is common between patients. If only autologous cells were used, then only patients with high initial white blood cell counts suitable for leukopheresis could be included in the trial, the number of immunizing doses for any 1 patient would be clearly limited, there would be the risk of accidentally giving viable autologous leukemia cells back to the patient, and cells from patients who did not go

into remission would be wasted. These conditions would severely limit the nature and extent of the trial. However, if allogeneic cells are used, then a single, suitable, highcount donor (who may die without going into remission) may easily provide enough cells (i.e., as many as 1000 ampuls, each containing 1×10^9 cells) for the long-term immunotherapy of several other patients. A large bank of cells may be rapidly collected for use in all patients in the trial.

In addition to immunizing the patients in this study with allogeneic cells, we gave frequent percutaneous (pc) BCG injections, because animal data from our Institute showed these injections increased the therapeutic response against the tumor (*4*).



TEXT-FIGURE 1.—Outline of the 4 arms of the trial (Barts I–III and Marsden I). *Thick, horizontal, black lines* indicate chemotherapy. *Arrows* represent weekly immunotherapy. All patients were in full remission at start of cytoreduction.

MATERIALS AND METHODS

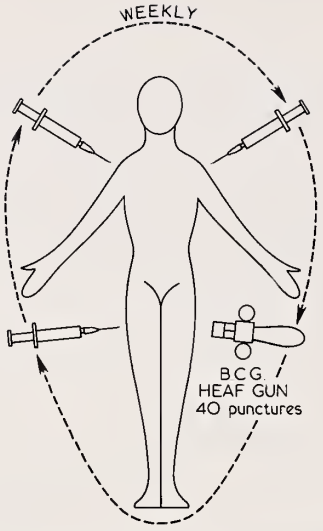
We designed our clinical trial as follows: Leukemia cells were collected with an NCI/IBM Cell Separator (*8*) from the peripheral blood of suitable patients with acute myeloblastic leukemia. Useful quantities of cells can be obtained from patients with peripheral blood leukemia in concentrations as low as 1000/mm³. As many as 1×10^{12} may be collected from a single donor if the blood count is high. We collected cells in this way from 65 patients (including some who had acute lymphoblastic leukemia), age 9–70 years; these patients suffered no discomfort during the 2- to 5-hour procedure. No deaths have been associated with cell removal.

The leukemia cells were sealed in 150–1000 glass ampuls, depending on the yield, with 10% dimethyl sulfoxide. They were frozen slowly (1° C/min) to −30° C with the use of a Gas Phase Programmed Freezer (Planer, Ltd.) and stored in liquid nitrogen. Each ampul contained approximately 1×10^9 cells. When required, the cells were rapidly thawed at 37° C, washed, and resuspended in medium 199 at 4° C. This process damaged only a few cells.

All myeloblastic leukemia patients presenting at the 2 units (including those who had undergone leukopheresis) were then included in the study. Text-figure 1 shows the 4 trial groups: 3 at St. Bartholomew's Hospital (Barts I–III) and 1 at the Royal Marsden Hospital (Marsden I). All patients were treated with rubidomycin and cytosine to induce remission as described in (*9*); there was a slight difference in the induction program in the 4 groups due to an attempt in Barts II and Marsden I to induce synchronous division of cells. The induction protocols were designed to minimize chemotherapeutic suppression of immunologic competence; thus they were neither intense nor prolonged, and we avoided using strongly immunosuppressive drugs. There was little doubt that the regimens we chose were good, since 40–60% of the patients achieved a full remission.

The essential goal of the program was to determine if immunotherapy during remission could favorably alter the natural course of acute myelogenous leukemia. The 4 arms of the study were designed to answer 2 questions. The first, most important question (Barts II and III) was whether immunotherapy had any effect on this disease. Our controlled study compared a group of patients receiving intermittent maintenance chemotherapy with a similar group of patients receiving identical chemotherapy plus immunotherapy. Any difference between these 2 groups would therefore, be due solely to the immunotherapy. Maintenance chemotherapy of these patients was designed to be short and intermittent so that it would not interefere with the immunotherapy. The second question (Barts I and Marsden I) was how the condition of a group of patients receiving only maintenance immunotherapy would compare with that of a group of patients receiving continuous, moderately intensive maintenance chemotherapy. By analyzing all 4 groups in this study, we hoped to obtain an indication of the best type of treatment for acute myelogenous leukemia.

Patients in remission were selected at random for immunotherapy. Only patients in full remission were immunized. As soon as full remission was achieved, all immunotherapy patients received BCG and irradiated allogeneic myeloblastic leukemia cells (plus monthly maintenance chemotherapy in Barts II and III) as indicated in text-figure 2. The cells were injected weekly into 3 limbs, and BCG was injected simultaneously into the fourth limb. The cells were irradiated with 10,000 R (with the use of a γ source) and then immediately injected into patients both intradermally (id) and sc. The total number of cells injected weekly was approximately 1×10^9 ml. The BCG (Glaxo Laboratories) was injected pc with a Heaf gun (40 punctures at 2 mm with a dose equivalent to 1×10^6 live organisms). The limb receiving the BCG varied from week to week.



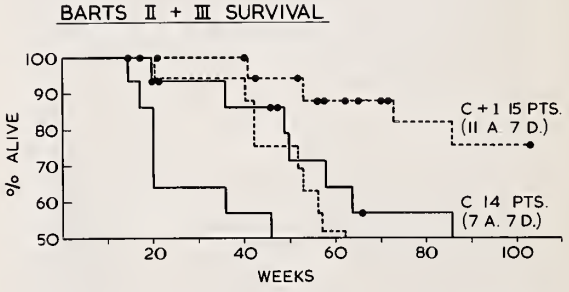
TEXT-FIGURE 2.—Method of weekly immunotherapy. Irradiated allogeneic myeloblastic cells were injected sc and id into 3 limbs. BCG was injected pc into the fourth limb. Weekly dose of cells was 1×10^9 and BCG, 1×10^6 live organisms. Each week BCG was given in a different limb; sites for the cells were rotated accordingly.

RESULTS

In June 1972 I presented in Paris (*10*) preliminary results on 46 patients in full remission studied for 2 years. Of these, 31 patients were given only maintenance chemotherapy of either continuous methotrexate and 6-mercaptopurine (Barts I) (*9*) or pulsed chemotherapy, given for 5 days every month, of cytosine plus either 6-thioguanine given for 5 days or a single injection of rubidomycin (Barts II and III). Actuarial analysis of remission duration of the 2 alternative methods of maintenance chemotherapy showed the same median remission length, 155 days. Twenty-four patients had relapses. In comparison, the actuarial remission duration analysis of the 15 patients given chemotherapy plus allogeneic cells and BCG revealed a median remission length of 430 days. Only 5 of the 15 patients had relapses. When this analysis was done, 2 of these 15 immunotherapy patients had not received maintenance chemotherapy, and both did well. One had a relapse at 520 days, and the other remained in full remission at 661 days.

This form of analysis has been criticized. The final assessment of the best way to treat patients with leukemia should not be measured in mean remission length, since increases of mean remission lengths (even of many months) may be a poor guide to better treatment methods. For example, 1 of the 9 immunotherapy patients who had a relapse in our study while receiving only immunotherapy was treated with the same drugs used initially to induce remission. He rapidly achieved a second remission which has been maintained by immunotherapy for nearly 2 years. His first remission duration would be no measure of the efficacy of treatment; the only real measure of treatment success would be the survival.

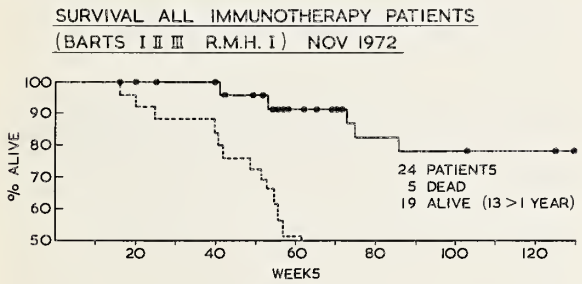
Therefore, we now present the survival data for this study, using life-table analysis of raw data. Text-figure 3 shows the survival curve (as of Nov. 1) for all the remission patients in the Barts II and III trials. The median survival for the 14 chemotherapy patients (7 are still alive) was 45–85 weeks. The median survival for the chemotherapy plus immunotherapy groups could not be determined because only 4 of the 15 patients have died. But clearly they fared much better than the patients who did not receive immunotherapy: If all immunotherapy patients had died the next day, the median survival would have been the same as that of the chemotherapy group; in actuality, most of



TEXT-FIGURE 3.—Life-table analysis of data, showing proportion of patients surviving against time and median survival time (50%): Comparison of Barts II and III chemotherapy alone (C) with chemotherapy plus immunotherapy (C + I). *Black dots* represent patients remaining alive. *Vertical drops* show when patients died. *Two lines* (one *broken* and one *continuous*) *without dots* represent the situation if all patients had suddenly died the next day (i.e., the worst possible results from each group).

the immunotherapy patients not only are alive but also remain in good health. It is probable that, in only a few months, analysis of these data will show that the worst possible results from the immunotherapy group would be better than the best from the chemotherapy group; at that time, a definite statement will be possible concerning this form of immunotherapy of acute myelogenous leukemia.

Separate analysis not shown here reveals that the 9 patients receiving only immunotherapy (Barts I and Marsden I) had survival figures similar to the 15 patients receiving chemotherapy plus immunotherapy (Barts II and III). Taking all 24 immunotherapy patients as a randomly selected group of myeloblastic leukemia patients (text-fig. 4), one can see that 17 patients lived $>$ 1 year (and 13 were still alive). Only 5 patients in the group had died. Median survival could not be $<$ 60 weeks; but the fact that 19 of the 24 patients remained alive and well, 3 for $>$ 2 years, makes it reasonable to suppose that many of them may become long-term.



TEXT-FIGURE 4.—Analysis, as in text-figure 3, of survival of all 24 immunotherapy patients so far included in the trial. *Broken line* represents the median survival if the 19 patients still alive suddenly died the next day. R.M.H. I = Marsden I.

Critical to an immunotherapy program is some measure to serve as a guide or rationale for the best possible immunotherapy in each patient. We previously described a serum factor (*10*) that may fulfill this role, but we do not yet know how those experiments relate to the management of patients. If we find a correlation between those studies and the clinical picture, then we can use the data to help achieve long-term survival for the most patients. This will require a trial with many patients, and we may have a long wait for the answers.

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DISCUSSION

B. G. Leventhal: Are there any questions for Dr. Powles?

S. K. Carter: Dr. Powles, in the Concord study, did every patient during relapse go on to similar secondary induction therapy?

R. Powles: Some 98% of the patients did.

Leventhal: Dr. Thomas will now give a short presentation.

J. W. Thomas: I shall present some data from the British Columbia Cancer Institute done in association with S. Landi from Toronto. We studied the maintenance of remission in a pilot group of patients with reticulum cell sarcoma and lymphoblastic lymphosarcoma.

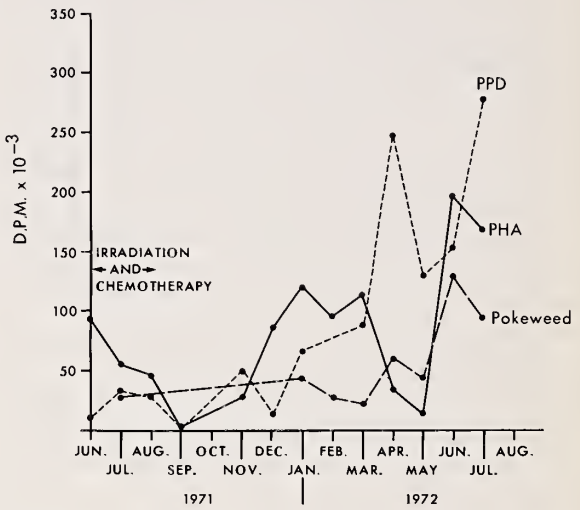
Seven patients with malignant lymphomas were studied. We chose this disease for two reasons: 1) It is highly malignant and 2) we believed that the BCG would have a reasonable chance of getting into the lymph nodes where much of the disease might be. Table 1 gives the diagnosis, the treatment, the incidence of recurrences, and the survival data. All patients were treated with irradiation and chemotherapy or chemotherapy alone. To be included in the series, they had to achieve a complete clinical remission.

The first patient had a recurrence in the cervical area. He had pain in the right arm and weakness in the right leg. He had 3 recurrences in the same area. The first recurrence was treated by irradiation and responded dramatically. The second recurrence was resistant to chemotherapy but again responded somewhat to irradiation. His final recurrence developed 3 months ago when he came in with pain in the same area. We watched him for 3 weeks. The tumor gradually worsened. Since there was a superclavicular node avaialable which appeared to be enlarging, we removed it. We teased out the lymphoblasts, irradiated them, treated them with neuraminidase, and put them back in with BCG. The patient was rapidly relieved from his pain. During the last 8 weeks, he has put on 8 pounds and returned to work 10 days ago.

The other patient who had a recurrence was a young woman with a superior mediastinal syndrome. She came to us after having been treated with irradiation and chemotherapy. She was only 5 weeks free of disease. She did not respond to the BCG vaccinations.

The BCG vaccinations were given weekly by multiple scratches rotated around the arms and legs. We continued to give the vaccinations weekly until we obtained a response. The vaccinations were then gradually decreased to every 2, 3, or 4 weeks. We used 40-mg vials of BCG which contained 12 million organisms/mg. We have been encouraged by these results. And as of June 1972, we have started a randomized clinical trial identical to the one I present to you here.

Text-figure 1 shows the results obtained from the third patient studied from the beginning of her treatment. Her phytohemagglutinin (PHA), pokeweed, and purified protein derivative (PPD) responses initially were sharply depressed during irradiation and chemotherapy, but at the present time, her PPD response is good and her PHA and pokeweed are both well within the normal range. This is typical of the reactions that we get.



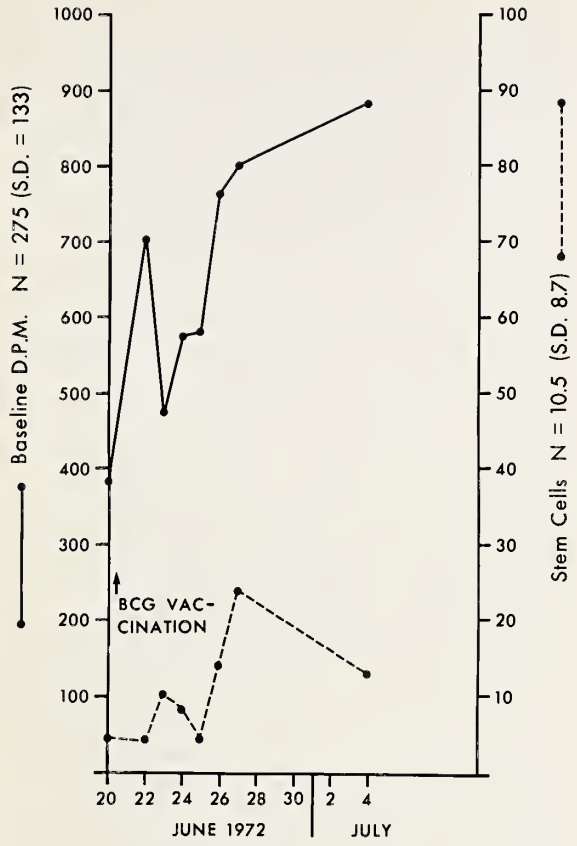
TEXT-FIGURE 1.—Lymphoma case #3: Mitogen and antigen monthly responses.

Text-figure 2 shows a daily study done after BCG vaccination in the arm. The upper line indicates the appearance each day of cells in the peripheral blood that will take up tritiated thymidine without stimulation. This is the so-called spontaneous transformation study. This line goes up for a little over a week—a little longer than the usual one—and then it comes down again. These cells are thought to be lymphoid.

At the bottom of text-figure 2 is another line, which represents peripheral blood colony-forming units. Almost invariably associated with the rise in spontaneous transformation is an increase in the number of these cells in the peripheral blood. These cells in colony are com-

TABLE 1.—Patients with malignant lymphomas: Treatment and survival data

	Lymphoma			
Diagnosis	Primary treatment	Recurrence	Months of survival	Comment
Lymphoblastic	Irradiation & chemotherapy	15, 24, 30 months	39+	Neuraminidase & BCG—fourth recurrence
Lymphoblastic	Chemotherapy	0	27+	
Anaplastic	Irradiation & chemotherapy	0	15+	General reaction
Anaplastic	Irradiation & chemotherapy	0	13+	
Anaplastic	Irradiation & chemotherapy	5 wk	9+	Off program
Reticulum cell	Irradiation & chemotherapy	0	9+	
Reticulum cell	Irradiation & chemotherapy	0	6+	



TEXT-FIGURE 2.—Daily study: Lymphoma case #3.

prised of both granulocytes and monocytes. We have wondered whether these monocytes possibly are important in regard to the armed macrophages which Dr. Alexander and Dr. Hanna have discussed.

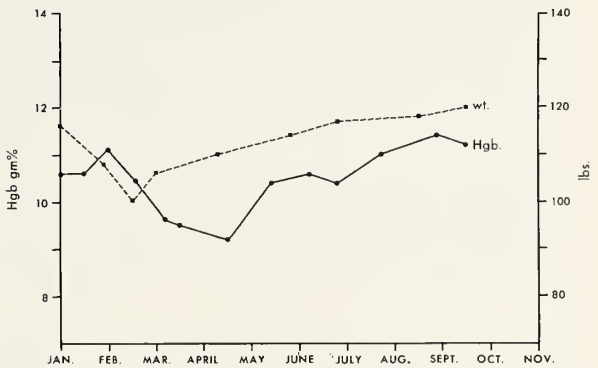
When you inject BCG into melanotic nodules, the number of these cells rises dramatically, sixfold to eightfold within about 5–7 days.

Patients with visceral melanoma (table 2) represent a terrible tragedy when they come in to see you. Our first 2 patients were treated with vaccinia, and they responded poorly. We elected to do 2 treatments with our next patients: 1) to transfuse them with blood to induce immunity because they inevitably seemed to be anergic to both PPD skin testing and BCG vaccination and 2) to give them oral BCG in an effort to put the BCG in where the tumor was.

Our first patient—i.e., the third entry in table 2—had no response. We didn't give her enough oral BCG and we gave her the treatment for only 2 weeks.

In 1 patient—the bottom entry in table 2—we were unable to convert his skin test or BCG response even though we gave him 5 transfusions from reasonably well-sensitized individuals. We gave him hundreds of milligrams of oral BCG and intradermal BCG. We did not obtain a bad response even though he appeared to be in great danger of a systemic BCG infection as he was totally anergic. Yet, when he came to autopsy, there was no evidence of this.

I'd like to elaborate on the clinical history of 1 patient (fourth entry in table 2). This patient is alive and well. Two months before I saw this patient, she came in with an intestinal obstruction from metastatic melanoma to the small bowel. She had two bypasses done. The tumor was not resected. It was a fair-sized mass at that time. When we saw this girl 2 months later, she had recovered uneventfully from the surgery, but her health started to deteriorate. Text-figure 3 shows her hemoglobin and her weight. We did 3 things with this patient. We had a female patient with subcutaneous melanoma who was being given vaccinia therapy and had also been immunized to BCG. We took 300 ml of blood from this patient, and you can see the little blip in the hemoglobin which we managed to get a week after we first saw this patient. We had immunized the patient the 1st day we saw her with intracutaneous BCG and also revaccinated her.



TEXT-FIGURE 3.—Case #4: Visceral melanoma.

TABLE 2.—Patients with visceral melanoma: Treatment and survival data

Stage	Treatment	Response	Survival (months)
Hepatic	Vaccinia	Poor	2½
Hepatic	Vaccinia	Poor	2½
Hepatic	Blood transfusion & BCG (oral)	Nil	2½
Nonhepatic	Blood transfusion, vaccinia, & BCG (oral)	Very good	9+
Hepatic	Blood transfusion 5 times, vaccinia, & BCG (oral)	Poor	3

Fortunately, both patients were A negative. We started immediately afterward to treat this patient. We gave her subcutaneous vaccinia for 5 weeks, which I'm not sure did her any good. We were looking for a general immune response. Then we started her on oral BCG. We gave her 5 mg at first, which was obviously not enough. We started to increase the dose and gave her 40 mg. Then we brought her in twice a week and gave her 40, 80, and finally 120 mg.

After 4 weeks, she again developed partial intestinal obstruction. By this time, she was getting abdominal pain over the tumor 24–48 hours after BCG administration. She settled down after this partial obstruction, and we lowered the dose of oral BCG, keeping it from then on between 40 and 80 mg each time we gave it to her. How much we gave her really depended on how much the pain had cleared up from the last time. She was a fine patient to look after. Each time she had just a classical delayed reaction of pain.

Furthermore, in addition to this large, fixed tumor mass over the left lower zone, as we continued to treat her, other tender masses became palpable during the next several months. The hemoglobin level gave us great concern, for it was very slow to return to normal. By April, quite clearly the tumor mass was receding. Presently she has a perfectly normal abdomen on palpation. She feels perfectly well. Her weight has gone up to where she tells me she doesn't want to go any higher; her hemoglobin has also come up.

Some of my other colleagues in Canada have been using oral BCG, which is perhaps worth looking into. Oral BCG is well tolerated. Calmette and Guérin originally gave their BCG orally to the children of Paris. I hope some of the animal experimenters here today will use this form of therapy.

Leventhal: Are there any questions for Dr. Thomas?

M. J. Mastrangelo: This is, to the best of my knowledge, the first we've heard today of a possible response of a visceral lesion. Was there any evidence, other than the weight gain, that the disease was going away?

Thomas: Yes. This patient had a large, fixed mass in her abdomen the 1st day she came to see us. The mass was in the left lower quadrant and actually extended across the midline.

Before we could detect any change in this mass, she started to feel better and her appetite returned to normal. And this mass entirely dissolved. I very carefully avoided saying—you will probably notice—that oral BCG eradicated the tumor, because we did take 300 ml of blood from the other patient with melanoma. But the patient from whom we took the blood still had recurrences of melanoma, and we have already used this patient again to produce the same result and didn't have any success.

The other possibility exists, of course, of a spontaneous regression. Spontaneous regressions have been reported. But we believed the pattern of pain 24–48 hours after the oral BCG was significant. At the present time we can give her only about 10–22 mg of oral BCG because she gets abdominal pain. We are, of course, in the dreadful position of not knowing whether to carry on.

F. C. Sparks: I thought it would be worthwhile to discuss both our results and complications with BCG. As you know, D. L. Morton first used intralesional BCG for treating malignant melanoma when he was here at the National Cancer Institute. That work has continued under his direction at the University of California at Los Angeles. His group's combined experience is now with >30 patients treated during the past 5 years (*see* table 3).

All 24 patients who could be sensitized to DNCB had regression of injected nodules. The PPD skin test converted from negative to positive in the 1 DNCB-negative patient who had regression of an injected lesion. In addition to the direct effect of BCG on the injected lesions, a systemic effect of BCG was manifested by the regression of uninjected nodules in 5 of 24 DNCB-positive patients. Biopsy of these uninjected, regressing nodules showed a lymphocytic infiltration. Thus BCG immunotherapy may control nondetectable, microscopic foci of tumor cells.

Five of the patients who have had complete regressions have been followed for at least 2 years; they have developed no recurrence. One of these patients has remained free of disease for 5 years.

Both the response rate and complications from intralesional injection of BCG may be dose related. Perhaps we are using a dose higher than that used by other people. These patients have received intralesional injections with

TABLE 3.—Immunotherapy of malignant melanoma with BCG: Results in 30 patients

		DNCB* positive	DNCB negative
		24 patients	6 patients
Regression of melanoma nodules:	Injected	24/24 = 100%	1/6 = 16%
	Uninjected	5/24 = 21%	0/6 = 0%
Survival:	>6 months	21/24 = 88%	0/6 = 0%
	>12 months	7/24 = 29%	0/6 = 0%
Free of disease:	>2 yr	5/24 = 21%	0/6 = 0%

* DNCB = dinitrochlorobenzene.

BCG on one or more occasions. Up to 10 ampuls of Lilly BCG have been injected into multiple tumor sites. Most patients received 2–5 ampuls. These injections were repeated in smaller doses after the patient became sensitized to BCG as seen by his response to the intralesional BCG injection. Currently, using the intradermal tine technique, we are giving concomitant, nonspecific immunotherapy with Chicago Research BCG.

The complications in these patients have been for the most part tolerable. As both Dr. Rosenthal and Dr. Pinsky pointed out today, systemic spread of BCG is the normal course of events. However, sometimes this systemic spread causes significant complications. Over half of these patients developed temperatures of >101° F and up to 106° F. The higher temperatures occurred in those patients sensitized to BCG. A flu-like syndrome occurred with marked malaise in 14 of these patients, significant tender adenopathy without suppuration in 3 patients, significant pain at the injection sites in 22 patients, and ulceration at the subcutaneous inoculation sites in 25 of 30 patients. In addition, 1 patient, who had received active immunotherapy with both his own irradiated tumor cells and BCG, had to be hospitalized for a week because of ulcerations on his chest wall at the injection sites.

Four patients had significant hepatic dysfunction resulting from BCG intratumor injection. Three of these patients were jaundiced.

One of these patients, a 30-year-old female who had undergone a radical groin resection 2 years ago, had negative nodes at that time and presented to us 1 year ago with satellite and in-transit metastases. In November 1971, she was started on both intralesional and nonspecific intradermal injections of BCG. Numerous liver function tests during that therapy gave normal results. Almost all the lesions given injections of BCG ulcerated and regressed in size. The uninjected lesions remained unchanged, neither regressing nor growing.

In March 1972, several new lesions were given injections of BCG. After 12 hours, her temperature rose to 104° F, and she developed a flu-like syndrome with nausea, vomiting, myalgia, and malaise, which persisted for 5 days. Two days after the injection of BCG, she became jaundiced. She initially refused hospitalization but then entered the hospital several days later, when her jaundice was clearing up. At that time her total bilirubin was 3.1 mg/100 ml; the direct bilirubin was 1.0 mg/100 ml; the serum glutamic oxaloacetic transaminase was 125 mμ/ml; and alkaline phosphatase was 850 mμ/ml. A liver biopsy at that time showed multiple, non-necrotizing, epithelioid granulomas but no acid-fast organisms. She made an uneventful recovery without therapy. Three weeks after BCG injection, her liver function tests gave normal results.

E. Klein: Dr. Sparks, did you ever treat any of the patients? We have seen reactions; maybe this sounds scary, but the temperature you know is going to go away. You can usually give the patients aspirin and the temperature will go down. If the patients are nauseated, you can give them some compazine, which is very often effective. And if you really get severe reactions, you could give the patients Compound F and some ephedrine, which usually helps.

Sparks: All 4 of these patients had granulomas in their liver biopsies. That in itself means nothing, since Gormsen [1] reported that 13 of 20 persons dying from various causes, 6 weeks to 40 months after BCG vaccination, had epithelioid cell granulomas in the liver. We elected to treat 2 of these patients because of malaise, daily high fevers, and the positive liver biopsies.

Klein: How did you treat the patients?

Sparks: We used INH (isoniazid), ethambutal, and streptomycin.

Klein: Did you use cortical steroid?

Sparks: No.

Klein: Since a good part of this is delayed-hypersensitivity reaction, let me assure you cortical steroid temporarily will do very little; for 3 days only the patient feels great.

M. R. Mardiney, Jr.: Gormsen, in his study, regarded the presence of granulomas in organs 2–3 years after vaccination as a normal sequel of BCG vaccination. He implied that a positive culture was the only way to validate clinical injection by BCG in such a group.

Sparks: We could not culture anything. The pathologists could not show us any tuberculous organisms in the liver. The patients on whom Gormsen reported were all asymptomatic; yet they had granulomas. I'm not sure that our 4 symptomatic patients had more granulomas than Gormsen's asymptomatic patients.

S. R. Rosenthal: Dr. Sparks, did any of these patients also receive allogeneic or syngeneic melanoma cells?

Sparks: Most patients received just intralesional and intradermal BCG. Those patients to whom you refer were receiving active immunotherapy with irradiated autologous or allogeneic tumor cells and BCG after surgical resection of all grossly detectable tumor.

L. Nathanson: Dr. Sparks, do you believe the clinical benefit accruing to these patients justifies the toxicity you saw in them?

Sparks: We now understand how to prevent some complications; so we don't expect to see as many in the future. There can be no question that the toxicity is justified by the results. Five patients have had complete regressions for >2 years, and one patient is free of disease for >5 years. All DNCB-positive patients have had regressions of injected lesions. We believe these patients have received significant palliation and have remained tumor free for longer intervals than they would have otherwise. A Phase III study is being designed to compare BCG with combination chemotherapy in this clinical study.

J. C. Kennedy: We found what you might call an encouraging type of complication of BCG therapy. We had a patient with a melanoma secondary in the brain stem

[1] GORMSEN H: On the occurrence of epithelioid cell granulomas in the organs of BCG-vaccinated human beings. Acta Pathol Microbiol Scand Suppl 111:117–120, 1955.

which was irradiated with some benefit. The symptoms dramatically worsened about 3 weeks later, and the tumor was irradiated again with no benefit. We put him on BCG therapy.

We removed the nodule, treated the cells with neuraminidase, and injected irradiated cells plus BCG subcutaneously. After the first injection, the patient developed a headache which lasted 6 days, nausea, vomiting, and other signs of increased intracranial pressure. We repeated the injections at 1-month intervals 3 times; each time we got the same effect. We then put him on oral BCG and maintained him for 5 months with no further increase in neurologic signs.

He went off oral BCG for about 3 weeks. He came back in poor condition, confused, and lethargic. We again gave him a subcutaneous injection, and this time he almost died. He developed a headache, nausea, vomting, stiff neck, and papilledema. Finally he went into a coma. We injected prednisone, which brought him out of the coma quite quickly. At the end of the week, he was much better. But in the meantime his liver had started enlarging; 2 months later he died.

Leventhal: Your point and Dr. Klein's point are well taken: We must not forget the delayed-hypersensitivity element in these lesions. We have seen fairly severe ulcers heal with prednisone in some patients who have received intradermal BCG. It's a two-edged sword, of course.

R. Weiner: I had 1 patient whom I treated on my immunoevaluation protocol. This patent had cutaneous metastases from breast carcinoma. She had previsouly had irradiation, and from my testing she was immunodepressed. I treated her with intralesional BCG. After the second administration—10 days after the first administration—she developed a fever of 106° F and was hospitalized. She developed disseminated intravascular coagulation. She was treated with heparin, INII, para-aminosalicyclic acid, and streptomycin. She improved transiently but died of cardiac arrest. We believe, although we had no cultures and no positive pathologic material, that this death was probably due to BCG given in the face of rapidly progressing disease.

Leventhal: Does anyone have a different type of toxic manifestation to bring up?

H. L. Sulit: Another complication, which would not be fatal, is the ocular complication of living BCG. Around 1954 among the Eskimos and Danes, I think, investigators noticed in BCG-treated patients a high incidence of pruritis and some other inflammatory condition in the eye which, if not controlled, could lead to blindness.

J. E. Sokal: Persistent infection has been documented. From some of our patients who are treated unsuccessfully with BCG for one or another advanced disease and who then die, we have occasionally cultured specimens of lung and liver. Recently we got positive cultures both from lung and from liver in a patient who died of malignant melanoma about 5 months after having BCG.

J. U. Gutterman: The intralesional administration of BCG appears to be associated with more toxicity than BCG administration by scarification. In addition to the patients whom I discussed today, we have 37 patients in whom we saw no major toxicity. We have seen overt low-grade fever and flu-like symptoms for a day or 2 after administration of BCG. We can confirm the experience of leukemia, about which Dr. Powles, Dr. Leventhal, and Dr. Weiner (presenting for Dr. Mathé) spoke—both in patients receiving chemotherapy and patients off chemotherapy given by the scarification route—the same low degree of toxicity. In addition, we now have nearly 50 patients with metastatic widespread melanoma who are receiving combination chemotherapy plus immunotherapy—primarily imidazole carboxamide plus BCG given by scarification. In this group we have many patients anergic to a battery of skin test antigens—that trial has been going on for >6 months now—and we have seen no evidence of disseminated BCG disease. We have seen only the low-grade fever and flu-like symptoms which occur a day or 2 later. So there seems to be a big difference between the toxicity effected by the scarification and the intralesional administration.

C. M. Pinsky: Several of our patients had pancytopenia for short periods after intralesional BCG treatment. The blood always returned to normal, but pancytopenia should be looked for. The reason we can't say anything with any assurance is that other patients had leukocytosis, which is not consistent.

R. S. Bornstein: We should be careful in using steroids in patients whose immune systems we are trying to stimulate. We don't know what effect steroids will have on their immune system.

Klein: That has been studied from the short-term—72 hours—point of view with practically nothing from the long-range point of view.

Sokal: I have also seen a case of pancytopenia and 2 probable cases of erythema nodosum of relatively short duration.

Leventhal: Dr. Rosenthal, why don't I let you be the transition person to the animal models? Go ahead and make your comments and tell us whether you think oral BCG is the preferable route of administration, particularly in the situation in which we are trying to treat visceral disease?

Rosenthal: I want to make one comment about giving prednisone to counteract toxic effects of BCG. I have given patients antihistamines for chills, fever, malaise, and joint pains and have obtained good results.

I have conducted numerous studies on oral BCG in animals and in humans, and the literature is full of such studies. Oral vaccination presently is used infrequently, mainly in Brazil. It is ineffective because, except in newborns, absorption is erratic and tuberculin conversion is very low. *Escherichia coli* and other organisms in the intestines are not absorbed normally; neither is BCG. Possibly if one had an open lesion in the intestine, BCG would be absorbed. Otherwise, theoretically, there should be no absorption.

When given orally, BCG may reach the crypts of the tonsils and be absorbed into the circulation through this route. Up to 5% suppurative lymphadenitis has been reported after BCG was given orally.

J. W. Thomas: Needless to say, we were concerned over the absorption. We scarified this patient on the leg, then waited a week, and gave her 80 mg of oral BCG; a flare-up effect was produced in the leg lesion which then developed into a real edematous mass within 24 hours. So whether BCG is found absorbed, I don't know, but it certainly creates delayed hypersensitivity.

Any patients now coming into our immunotherapy clinic automatically get a liver and spleen screen. If you are going to try to attack liver metastases, you must do so when they are very small and you perhaps have some chance of getting at them.

Also, in the past few months, we have simultaneously administered oral BCG and injected subcutaneous nodules. The effect seems to be augmented by the subcutaneous nodules when they are injected. We are now injecting as little as 0.05 mg of BCG into subcutaneous nodules which produces a striking local effect if the oral BCG is given simultaneously.

M. Chase: Dead BCG will give up to their environment soluble proteins, and using a dose of 80 mg certainly one has no problem of having to find a barrier or a defect.

Nathanson: In regard to intrahepatic metastases, if the object of oral BCG is to bring the organism in juxtaposition to the tumor cell, this is hardly the best way to do it, as it's well known that such metastases derive their blood supply almost entirely from the hepatic artery, not from the portal circulation.

Leventhal: Let's talk for a moment about the animal models.

B. H. Waksman: There's a long-standing tradition in immunology to make a distinction between immunization by way of the bloodstream and intradermal immunization which goes almost entirely to draining lymph nodes. The evidence began in the 1920s with immunization of pneumococci and streptococci; also later work suggested that, with the intravenous route, the primary response comes from the spleen and is largely antibody. In almost all the situations in which this was seen, some cell-mediated sensitivity was not seen. When these observations were made, there was no explanation for that event.

More recent work—and this, believe it or not, also includes some work on tumors—strongly suggests that immunization directed to the spleen induces preponderantly antibody formation, not cell-mediated immunity, probably by way of an enhancement mechanism.

But immunization directed to the nodes, usually in very small doses and either intralymphatic or intradermal, induces pure cell-mediated immunity with or without some additional degree of antibody formation.

This is really one of the important elements that was not pinned down in what we heard this morning. Someone remarked in passing that giving BCG directly into a melanoma lesion is really giving a systemic dose—the equivalent of an intravascular dose. But giving BCG directly intradermally is really giving an intradermal dose. The effects of the 2 ways of administration might be expected to be entirely different.

Leventhal: There is another problem associated with the route of administration. It seemed to be generally agreed that administration by scarification is less toxic systemically. The difficulty with scarification is that one must be sure of the dose actually administered to the patient. Does anyone have any solutions for this problem?

Rosenthal: There was a study comparing the absorption of BCG when given by the multiple puncture scarification method and intradermally with the use of tagged BCG. The recommended doses for the multiple puncture administration and for the intradermal administration were compared. Four times as many organisms were absorbed by the multiple puncture method compared to the intradermal method.

Leventhal: Some of these tagging studies might be interesting to clinicians.

Nathanson: Dr. Rosenthal, does your comment apply equally well to scarification and multiple puncture? Are multiple puncture and scarification essentially the same thing biologically?

Rosenthal: Yes. The method I first described was multiple puncture, but it was modified by Negre into scarification. The results by the 2 methods were identical concerning immunization in guinea pigs, tuberculin conversion, etc.

Nathanson: In multiple puncture, is blood drawn?

Rosenthal: A little, sometimes.

Nathanson: Scarification, as I understand it, usually does not produce bleeding.

Rosenthal: If you do it right, it does.

Leventhal: Dr. Ziegler and I have discussed this, and scarification seems to produce bleeding in white skin more often than it does in black skin. This may have something to do with the thickness of the skin and also with the health of the patient.

If your patient has had a lot of chemotherapy, particularly a lot of steroids, aren't they going to have thinner skin, Dr. Klein?

Klein: Do you have statistical data?

Leventhal: The skin feels thinner.

Kennedy: Perhaps oral BCG is equivalent to internal scarification. You have the whole digestive tract available for entry of BCG.

S. D. Chaparas: Dr. Rosenthal ought to make it clear, though, that the doses used for the percutaneous application are a couple of logs greater to start with, and this is why you may get more organisms.

Nathanson: No, he meant equal number of organisms.

Rosenthal: No, equal clinical doses. It was the recommended clinical dose for the multiple puncture method and the recommended clinical dose for the intradermal method.

Sokal: But the recommended clinical dose for the multiple puncture method is much higher?

Rosenthal: The concentration of vaccine is higher, but a larger dose is not necessarily injected.

Leventhal: Dr. Rosenthal, is there a reference on the method and the procedure utilized to use these tagged bacteria?

Rosenthal: Yes.[2]

The principle of the multiple puncture method is that BCG must get into the cutis where there are blood vessels and cells of the reticuloendothelial system (RES). BCG does not work in the epidermis. This is the difference between the multiple puncture method as I first described it and the multiple pressure method for vaccination against smallpox. In the latter method, one barely pricks the epidermis. The virus will multiply in the epidermal cells. But with BCG one must go deeper into the cutis; therefore, one may get a little bleeding.

Sparks: Dr. Rosenthal, would you expand on that? If one were to, say, give 0.1 ml of the Chicago Research BCG intradermally, what would the total organisms in liver be compared to, say, a half cc of the Chicago Research BCG given by the tine method as you prescribe it? How do those doses compare? These are probably the clinical doses that the different groups are giving today.

Rosenthal: The dosage for the intradermal method is approximately half a million to a million organisms or clones, and I believe that, by the multiple puncture method, absorption would be 3 or 4 times that much, as shown by the tagging method.

Sparks: Is that delivered to the node, to the bloodstream, or where?

Rosenthal: The organisms are delivered to both the node and the bloodstream. When injected transcutaneously, the organisms follow this route.

Leventhal: Would anyone like to comment on schedule, in terms of design of clinical trials?

J. L. Ziegler: The schedule and dose are probably closely related. Studies done in Uganda a year or two ago by A. Z. Bluming and his colleagues showed a difference between Glaxo BCG and Pasteur Institute BCG given at a difference of about 3 logs of dosage over the same period of time.

A group of 6 patients with malignant melanoma treated with the Glaxo BCG had no potentiation of their delayed-hypersensitivity responses. But 6 additional patients treated with the Pasteur Institute BCG given by scarification all had significant potentiation of their delayed-hypersensitivity responses.

Although this study isn't quite related to schedule, it does involve dosage and host response to the material.

D. W. Weiss: A vital point to be made about schedule is the necessity of monitoring carefully, by a variety of tests, the immunologic status of the patient. In some preliminary clinical trials of the methanol-extraction residue (MER) that we have been conducting in patients with acute myelocytic leukemia, we found that, within 6–8 weeks after the last MER treatment, cellular immune reactivity sometimes began to decrease; within a few weeks after that, some patients had relapses. However, when we maintained treatment with MER, immunologic capacity remained high and those few patients have remained in remission so far, some for as long as 24 and 26 months.

The only way to predict what schedules will be most successful in a given patient with a given neoplastic illness is to conduct ongoing immunologic monitoring in the hope that some test will give early indication of clinical changes.

Klein: We find that the skin test is a reasonably good indicator of responsiveness, and it seems to decline within 2–5 days after BCG administration as we quantitate it. It dovetails somewhat with some animal data about which Dr. Alexander told me..

So our schedule is to continue giving BCG at 3-day intervals, if the patient will take it, and at 5-day intervals (or as close to this time as possible).

Furthermore, you have to titrate the concentration of the PPD that you are going to use, because your arm can fall off, and you want to maintain a high level of responsiveness with as little side effects from BCG as possible.

I. Djerassi: As you may recall, 3 years ago we developed a new process for harvesting large amounts of granulocytes from single normal donors. We used filtration of the blood instead of sedimentation. The granulocytes are separated from other blood elements by reversible adsorption on nylon fibers. More recently we developed and constructed an apparatus for mechanical and automated extracorporeal circulation and filtration of the donors' blood. This apparatus enables us to increase the yield of granulocytes obtainable on single donation from 40 billion cells by the manual method, to an average of 100 billion cells.

This machine was not built for separation of macrophages nor for the separation of cells, as with the IBM-NCI cell separator which is a continuous-flow centrifuge, not particularly efficient for the separation of granulocytes.

A few months ago we recognized that the improved preparation of granulocytes harvested from normal donors did indeed contain significant numbers of monocytes. Using careful differential counts and special histochemical stains for monocytes, we found that up to 7% of the total cell population in a typical concentrate were monocytes. Although this percentage was not very large, if one considers the total amount of cells harvested, it amounts to 7 billion monocytes in each cell concentrate containing 100 billion granulocytes. We also realized that patients with acute leukemia or solid malignant tumors, during transfusions with up to 100 billion granulocytes given during an infection associated with profound leukopenia, had received substantial total amounts of normal monocytes—up to 70 billion.

Usually the peripheral blood following leukocyte transfusions showed increased numbers of both typical and atypical monocytes in these patients; at the same time, what appeared as unusually effective responses to the administered chemotherapy was seen. Most of the transfused cell concentrates had been obtained from HL-A random donors, and symptoms suggesting mild graft-versus-host disease were observed. Increased levels of mono-

[2] Spiess H, Poppe H: Weitere Untersuchungen mit radioaktiv markierten Bakterien im Rahmen der Tuberkulose-Schutzimpfung. Klin Wochenschr 32:186–187, 1954.

cytes and blastoid-like cells in the peripheral blood might, therefore, have been derived from the recipient due to a reaction to incompatible cells, rather than to the circulation of the transfused monocytes. The favorable outcome of the infections and the concurrent regressions of the tumors in these patients suggest that transfusions of large amounts of monocytes do not result in unfavorable effects.

Currently our efforts are directed toward increasing the monocyte counts of the donors before leukocyte donations and toward separating monocytes on a large scale from the granulocytes. Clinical studies on transfusion of monocytes stimulated in vitro with an activator (e.g., concanavalin A) and in vivo or in vitro with specific antigens in the presence of sensitized lymphocytes were initiated jointly with Dr. Klein and his associates. Transfusions of monocytes so treated also seem to be feasible without serious side effects in the recipients.

Leventhal: I have tried unsuccessfully to get someone to discuss animal models and scheduling. One reason for my lack of success is that there are few animal experiments in which immunotherapy has been combined with the type of conventional chemotherapy or radiotherapy that patients must have. Dr. Weiss did show us some data with MER and radiotherapy yesterday; these do point out a need that all the clinicians have, from people who have a successful immunotherapy model. We need to know how the usual drugs that we use in treating human tumors affect the immunotherapeutic response.

R. L. Simmons: Did you want such a model? We have such a model without BCG.

Leventhal: No, what I wanted was a model, which we seem to have in patients, indeed perhaps with Dr. Powles' type of investigation, where it would be possible to combine immunotherapy with more conventional therapy. This is the type of model I was looking for.

Simmons: Do you want BCG?

Leventhal: Yes. But I'll settle for MER.

Weiss: Y. Stupe in our laboratory is now involved in some fairly extensive studies to determine whether MER and BCG (comparatively) can reverse the immunosuppressive effect of prolonged administration of cyclophosphamide, methotrexate, and other chemotherapeutic agents in several species. The available results suggest that, at least under some parameters, this can be done. So this does facilitate using higher levels of chemotherapy in the animal models than would otherwise be feasible, without causing severe difficulty to the animal from infection.

So conceivably one of the major fringe benefits—which may not be a fringe benefit but a core benefit—of supposedly nonspecific immunostimulation may be the potentiation of conventional therapy. This should not be ignored.

Waksman: The animal model has something very important to suggest, which hasn't been mentioned at all at the meeting: All the adjuvant functions of tubercle bacilli are supposed to reside in wax D. I haven't heard anybody here speak of attempts to use wax D, which is free of tubercle bacillus antigens, to change the behavior of the tumor.

We certainly learned from the Rapp-Zbar experiment that some evidence exists for actual sensitization against antigens of the tumor. If that is what is being observed, one is concerned with the adjuvant effect of the injected material. On the other hand, some of the other work has strongly suggested we are injecting BCG to actually achieve sensitization against the antigens of the BCG itself. This should be the number-one question you'd like to answer because you want to know which effect it is you're after.

A problem with wax D is that it is perhaps not in the best physical form to achieve its effect. Although we have just heard about oil droplets with Dr. Ribi's preparation, Dr. Ribi's preparation still has tuberculin antigenic activity. If you use wax D, you'd also want it in some form like on the surface of oil droplets.

Unidentified speaker: It dissolves in oil?

Waksman: Yes. And I would strongly suggest that this should be tried.

Mardiney: The AKR model, in two aspects, probably represents one of the best challenges in terms of immunoprophylaxis, chemotherapy, and immunochemotherapy. In the AKR model, the mouse is an intrinsically poor responder both to tumor virus and to tumor antigen; but indeed it responds. Thus one way or another we could use this model to monitor the various approaches, in terms of immunoprophylaxis and chemotherapy when a natural tumor is present, immunotherapy when a natural tumor is present, and combinations of chemotherapy and immunotherapy to enhance an animal which is an intrinsically poor responder.

Now, the question is: How often does the model fit in with the human situation? It may or may not fit in most of the human situations. But it is a combined model set up perfectly for attack.

J. G. Bekesi: J. Holland and I have been investigating Dr. Weiss' MER preparation on an AKR model. We have diagnosed spontaneous leukemia in AKR mice. We then treated it with various chemotherapeutic agents, after which we immunized the mice with MER. At present we are running around 110 days after the diagnosis. With the therapeutic group alone, all the mice died by day 65. Single or multiple immunizations of MER up to the dose of 0.5 mg per single injection resulted in about 20–30% of animals free of disease at day 110. But when we used larger doses, such as 1 mg or 5 mg of MER, we have seen enhancement; i.e., the animals died at a faster rate than those receiving drug therapy alone.

Sokal: I would make a plea for some of us immunotherapists. It is not entirely clear to me what is gained by giving a dose of BCG every other day. BCG doesn't act that fast.

Certainly we have been able to maintain a high level of cellular immune responsiveness in our patients with a maintenance schedule of anywhere from 3 to 4 injections a year, which is much more tolerable. And we get to this level of reactivity with an induction schedule which, at worst, is about once a month.

Would some of the basic scientists who have studied the time sequence of events after BCG is introduced into the skin of the animal explain to me what is a proper schedule to get cumulative effects? I don't think BCG administration needs to be scheduled every other day.

Rosenthal: I made the point before that, when one discusses the ideal schedule for a specific disease, e.g., tuberculosis, the dosage differs from that of a nonspecific disease, e.g., malignancy. A large antigenic dose is necessary for a nonspecific effect, and the antigen must be injected frequently. The RES is stimulated after BCG administration, but this stimulation regresses in a month or so. If specific antigen is reinjected, the RES is quckly restimulated. In tumor therapy, BCG must be injected repeatedly to maintain a stimulated RES. In tuberculosis, a relatively small dose is effective. With reinfection, an amnestic reaction activates the RES.

Klein: We all favor the inbred, carefully predictable kind of animal model. But let us remember that man is an outbred model and totally unpredictable. We do have autochthonous tumors ad infinitum available in large domestic animals. In New York City alone, they are killing 300,000 cats with tumors. They are killing about 200,000 cows with tumors in the United States. These are beautiful models with which to work. Instead of having to play around with tail veins, you can get liters of white blood cells, and you can get kilos of proteins.

You do have to look at large models in autochthonous tumors as they occur in man. So let's get all the detail data in the inbred animal. And let's not forget about this enormous reservoir of animals.

Leventhal: Is there some specific point on schedule that anyone wants to bring up?

Chaparas: With regard to the best route, dose, and strain to be used in man, the simple answer is we don't know because these studies haven't been done.

Dr. Ziegler referred to the use of Glaxo BCG given intradermally, which has about 1 million organisms. He compared this to the Pasteur strain BCG given by scarification, which has organisms a couple of logs higher. We don't know what gets in.

To study and compare strain differences, one can administer equivalent doses given by the same routes. You can give the Glaxo strain—they do make a stronger product and they do have one for percutaneous application. It would have been ideal to have compared Glaxo and the Pasteur strain by the scarification route or by the intradermal route and to have found the optimal doses.

Until systematic studies are done, first in animal and then in man, we'll just be fencing with windmills.

Weiss: In >13 years of work in many thousands of inbred mice, outbred mice, and guinea pigs with a variety of tumors and infectious diseases, I have never seen a single instance in which >2 injections of either living BCG, phenol-killed BCG, or MER were more effective than 1 or 2. I have seen instances in which 2 are better than 1. But beyond 2, over periods of >1 year in a host-parasite relationship, I have never found repeated injections to be significantly more beneficial.

On the other hand, clinical trials that we have begun indicate that repeated intradermal injections of MER in the acute myelocytic leukemia patients are necessary for immunostimulation or therapeutic activity. There indeed may be a wide gap between mouse and guinea pig and man.

Sokal: How often do you inject the agent into man?

Weiss: Once a month.

Sokal: I concur with you.

Waksman: In the 1920s delayed hypersensitivity with streptococci was achieved without doubt much more effectively with multiple, repeated, very small, intradermal injections than with a single injection. Also, in some of the work on autoimmunity in the last decade, there turned out to be a number of instances where antigenicity was really of a very low order, and a single immunization did not produce autoimmunity. This is the case, for example, with so-called insulinitis, which produces pancreatic island lesions.

Tumor antigens are generally very weak, and this is the basis of some of the remarks made yesterday that, when you work with transplantable tumors, you are not really using a tumor that's comparable to the autochthonous tumor in man. Autochthonous tumors have extremely weak antigenicity. And if they are at all like these weak tissue antigens about which I speak, undoubtedly repeated immunization—here I would disagree with Dr. Weiss—with as many as 3, 4, or 5 will have continual, increasing effectiveness in producing cell-mediated hypersensitivity.

Another related point worth making is that the reinjection of whole tubercle bacilli into the individual who already has tuberculin sensitivity induces a tuberculin reaction—or what they used to call a "cloak" reaction at the site of the new injection—and invalidates the injected material to a large extent. And it would be highly desirable, for this reason as well, to use the adjuvant alone, if that's what you're after, and not the whole tubercle bacilli.

Leventhal: I'd like to move on to immune evaluation, because it seems that, in many of the models we have discussed, the best way to assess the amount of drug or the amount of immunotherapy being given to the subject, be it animal or human, is the change in the effect on the immune response. Dr. Herberman will begin the discussion.

R. B. Herberman: There are 2 comments I'd like to make that separate from each other.

The first is that a number of people today have made a major point that, in clinical studies, it is highly desirable not just to do the empirical trials but also to monitor the patients to get information about what is happening to their immune response during the before-and-after treatment with BCG or other immunotherapy.

One thing that I have particularly noted with the animal studies is the lack of the use of monitoring techniques. It is important to ask the questions in just the same way as the clinicians are so that we can correlate the information from animal experiments with that ob-

tained in the clinical area. This is just a plea for immunologic monitoring to be incorporated as much as possible in the animal model systems that are studied.

The other comment is about one clinical study. We have been involved in immune evaluation of patients with acute leukemia in studies that Dr. Leventhal's group has been doing. Compared have been 2 in vitro assays, Dr. Leventhal's assay of lymphocyte stimulation and assays in our laboratory of cytotoxicity in vitro. Also skin tests were done with extracts from the blast cells. Although reactions were positive at various times with all assays, clearly only the skin tests correlated with the clinical state of the patient.[3] Just having an in vitro assay or one parameter to follow may not be sufficient. Real questions exist as to whether a particular assay will be relevant.

To perhaps put the skin testing—detailed this morning by Dr. Leventhal—in a bit more perspective, I want to mention some of the results that we've obtained on skin testing with blast cell extracts in acute leukemia. We tested patients with acute lymphocytic leukemia (ALL). When tested with membrane extracts prepared from the leukemia cell membrane, most patients studied in remission gave positive delayed-hypersensitivity reactions; whereas most patients tested in relapse did not give positive reactions. The reactions appeared to be in response to tumor-related antigens; comparable preparations of remission cell membranes did not give reactivity.

Similar results have been obtained in acute myelogenous leukemia (AML), with high reactivity seen to blast cell membranes in remission and significantly lower reactivity in relapse.

We also did serial studies on 3 patients, 1 with ALL and 2 with AML, on whom skin tests were done at various times. By our criteria of a positive reaction being ≥ 5 mm at 48 hours, these patients, and a number of others, gave reactivity when they were in remission, lost their reactivity in relapse, and then became positive again once they went back into clinical remission. So testing with autologous extracts may very well reflect what's going on in the patient.

In contrast, the allogeneic preparations with which we have done some testing and which are the main components in the data that Dr. Leventhal presented this morning, have surprisingly given a low incidence of reactivity. The amount of reactivity of the leukemic patients to allogeneic extracts has been much lower than that of leukemic patients to autologous extracts; although 19 of 31 patients with ALL responded to ALL blast extracts at one time or another, this is much less than we would have expected or seen with the autologous preparations. In addition, most of the patients were tested simultaneously with 3 or 4 different preparations and generally responded to only 1 of those preparations. So the amount of cross-reactivity that we are seeing by this test is not extensive.

[3] Leventhal BG, Halterman RH, Rosenberg EG, et al: Immune reactivity of leukemia patients to autologous blast cells. Cancer Res 32:1820–1825, 1972.

Ziegler: I'd like to add to Dr. Herberman's remarks. It has also been our experience in Burkitt's lymphoma that the skin test reactivity does correlate well with the clinical course of the patient.

But I would like to warn and perhaps again plea with other clinicians working with skin tests. We have now had experience with thousands of skin tests in children. Our patients are all black. In black skin, it is more difficult to read induration than in white skin. But, nevertheless, my plea is that the skin tests be done by 1 individual, read by another individual who has no knowledge of the previous skin tests and their site of implantation, and read at 24 and 48 hours. This is something that Dr. Herberman has suggested and should be emphasized because we have found that skin tests may become positive at 24 hours and then become negative at 48 hours. There may be some significance to this type of reactivity, which has to be observed in as careful a scientific manner as possible.

G. W. Santos: I knew about the initial correlation of skin testing with Burkitt's lymphoma. Not too long ago there was a discussion at a meeting about the falling down of the theory of skin correlation of Burkitt's tumor. Dr. Herberman mentioned the data were not holding up, and now Dr. Ziegler comes back from Africa and says they are holding up. What is the current status of skin test correlation with Burkitt's lymphoma?

Ziegler: You might be referring to malignant melanoma, in which we did report a very preliminary study that initially revealed a positive correlation with the extent of disease which, however, did not hold up on an extended study. But the Burkitt's lymphoma data have held up through 2 or 3 years of observation.

P. Alexander: I find Dr. Herberman's results very puzzling, because when we took irradiated autologous blast cells and injected them into patients in remission, we never saw in a series of about 40–50 patients with AML and ALL combined the slightest sign of any skin reaction. In our hospital 450 injections of irradiated autologous tumor cells have been made, and we have seen only 3 skin reactions out of that whole series with the patient's own irradiated cells.

Apparently preparations from cells and the whole cell differ considerably. Of course, one additional feature is introduced whenever one prepares cells: the danger of contamination with bacterial materials that introduces cross-reactions as an artifact in these tests. And this is, of course, going to be most serious when one uses the autologous materials, for, there, one is likely to have the contaminant to which the patient is sensiive.

In London Dr. Hughes studied such extracts some years ago. He started out with 50% positivity, which then went down to 30%; his latest claim is that the positivity is down to 7% as he is becoming more careful and more sterile in the preparation of his material.

Leventhal: With that dash of cold water, I'd like to call on Dr. Seigler who is going to tell us about immune evaluation of some of his patients with solid tumors.

H. F. Seigler: This is a preliminary report from the standpoint that this study has been ongoing for 3 years.

I can give you more complete data on some patients and less complete data on others. This study is on melanoma patients. Presently, 140 patients are being treated with immunotherapy.

I'd like to give you our plan, our reasons for the study, and whether or not we are accomplishing the objectives.

Immunotherapy Plan

Stage I—The patient is sensitized with BCG—an effort to sensitive the patient to a specific but indifferent, perhaps cross-reacting, antigen.

Stage II—The patient receives intratumor injection of BCG—to produce a local delayed-hypersensitivity response in the tumor nodule.

Stage III—Autologous lymphocytes (5×10^9) obtained on the blood cell separator are specifically sensitized in vitro and administered to the patient—an effort at accomplishment of adoptive transfer to convey specific immunity.

Stage IV—The patient is boostered with X-irradiated autochthonous tumor cells that have been treated with neuraminidase. BCG is used as an adjuvant—an effort to get specific immunity or specific delayed hypersensitivity.

Unidentified speaker: In Stage III, are the autologous lymphocytes sensitized to BCG or tumor?

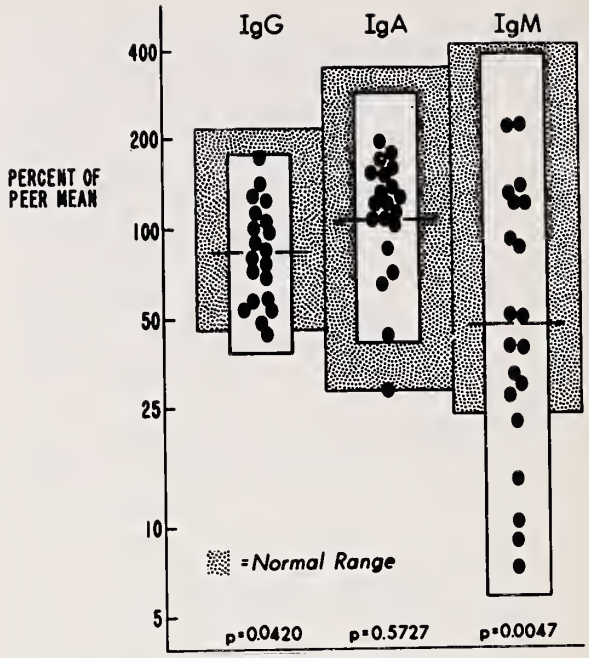
Seigler: They are sensitized to tumor, X-irradiated autochthonous tumor cells that have been treated with neuraminidase.

It is quite important to know what the prior treatment of the disease has done to the immune status of the patient and then to evaluate with each stage of the immunotherapy plan—these stages being between 6 and 12 weeks apart—what each stage will sequentially alter in the observed things we're testing.

Table 4 lists the skin test antigens that have been studied in our institution in 150 nonallergic control patients; all the patients are studied with these antigens.

We ran serum immunoglobulin levels in all patients (text-fig. 4), and 5 of the 22 patients demonstrated low IgM.

We tried to establish in all our patients specific humoral immunity (table 5). This was done by 3 techniques: mixed agglutination, cytotoxicity, and immunofluorescence. Table 5 shows that 6 of 9 patients were positive. I will expand on this observation later.



TEXT-FIGURE 4.—Serum immunoglobulin levels in patients with malignant melanoma.

The result of the skin tests (text-fig. 5) showed that this population of patients was hyporesponsive to the skin test antigens listed in table 4.

We tried to monitor cellular immunity also by a variety of techniques. Each patient was studied before and then sequentially with the agents on the left, the stimulating antigens, and then was studied at each stage.

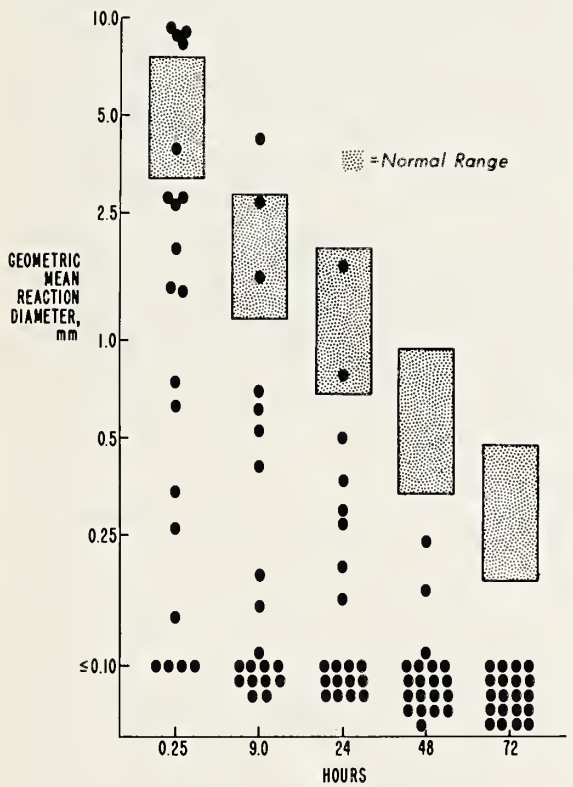
I mention this because, of our initial group of patients, the 7 patients who completed all 4 stages of immunotherapy initially responded to PHA only; although impaired, the response was good.

TABLE 4.—Antigen panel for evaluation of immune competence

Antigens	Dilutions	Antigens	Dilutions
Alpha streptococcus	10^{-4}	PPD	0.002 mg
Haemophilus infiuenza	10^{-4}	Blastomycin	10^{-3}
Neisseria catarrhalis	10^{-4}	Histoplasmin	10^{-3}
Staphylococcus epidermidis	10^{-4}	Coccidiodin	10^{-3}
Proteus mirabilis	10^{-4}	Oidiomycin	10^{-3}
Pseudomonas	10^{-4}	Trichophyton	10^{-3}
S. aureus	10^{-4}	Alternaria	2×10^{-3}
Enterobacter	10^{-4}	Aspergillus	2×10^{-3}
Escherichia coli	10^{-4}	Hormodendrum	2×10^{-3}
Pneumococcus	10^{-4}	Rhizopus	2×10^{-3}
Enterococcus	10^{-4}	Mumps	Undiluted
Mixed respiratory	10^{-1}		

TABLE 5.—Serologic evaluation for tumor-specific antibody, TSA-seropositive responses in 9 of 22 patients

Patient	Mixed agglutination	Cytotoxicity	Immunofluorescence
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	−
6	−	−	+
7	−	+	−
8	−	−	+
9	+	−	Not tested
Number of patients positive/ total	6/9	6/9	6/8



TEXT-FIGURE 5.—Cutaneous hypersensitivity reactions in patients with malignant melanoma.

Table 6 shows the stimulation observed after patients completed all 4 stages. In addition to demonstrating that one could regain, at least by this measure, lymphocyte responsiveness in vitro, table 6 shows that the PHA response was normal for our laboratory. To unrelated, mitomycin-treated lymphocytes, the response was a bit low: an average stimulation index of 14.

TABLE 6.—Stimulation indices in 6 patients completing immunotherapy

Stimulating antigen	Average stimulation index*
PHA (20 μl of 1:10 dilution)	90.0
Unrelated lymphocytes†	14.0
Autologous melanoma cells‡	8.5
PPD (50 μl/2 ml)	4.0
BCG (100 μl/2 ml)	2.5
Unrelated fibroblasts‡	2.2
Autologous lymphocytes†	1.0

* The degree of stimulation in each test was expressed as a ratio between the mixture and the appropriate control, called the stimulation index.

† Mitomycin treated.

‡ 5000 R radiation.

The autologous melanoma cells treated with 5000 R were interesting because of the stimulation index of 8.5, the highest index we've observed in studying any type of malignancy in patients or transplants in patients. This gave us some in vitro evidence that at least autologous melanoma cells could stimulate the patient's lymphocytes in culture.

The PPD and BCG were about standard for our laboratory. The unrelated fibroblasts and autologous lymphocytes were also near normal.

We also studied cellular immunity by cell-mediated cytolysis of a radiolabeled tumor monolayer (table 7). We did this by 2 techniques: using tritiated thymidine and using ^{125}I uridine.

There were 6,200 counts per minute of the monolayer. This was with tritiated thymidine. In tumor plus the patient's cells (this is an autochthonous tumor), cytolysis to 1,700 counts per minute of the monolayer could be seen. Tumor plus an unrelated cell revealed minimal killing. This was probably the allogeneic HL-A effect. The fibroblasts and controls were normal.

One has to remember that, to interpret these data, one has to stay in an autologous system; otherwise, one will be dealing with syngeneic inhibition. Also, one has to use very early monolayers because we've demonstrated, as

TABLE 7.—Cellular immunity

Target cell*	Counts per minute
Tumor (autologous)	6,200
Tumor + A cells	1,700
Tumor + X cells	4,800
Fibroblast	21,000
Fibroblast + A cells	17,200
Fibroblast + X cells	20,500

* Radiolabel, tritiated thymidine. A cells, patient lymphocytes; and X cells, unrelated normal lymphocytes.

have I. and K. E. Hellström, that after about 9–15 passages the tumor-specific antigen disappears; so one can't use continuous long-term culture lines.

Table 8 shows additional data on 70 patients tested for antimelanoma antibody by the 3 techniques I referred to before. Of the 70 patients, 63% of them were positive by at least 1 technique, and 37% were negative by all techniques. Our control sera were negative by all techniques.

TABLE 8.—Serologic evaluation for tumor-specific antibody, TSA-seropositive responses in 31 of 70 patients

Technique	Number of patients positive/total No. of patients
Mixed agglutination	17/31
Cytotoxicity	30/31
Complement fixation	20/31

Some specificity studies—and obviously a lot more need to be done in this area—revealed that all positive sera remained positive after absorption with HeLa, HEp, 3 normal skin fibroblast cell lines, and normal lymphocytes that included all the defined HL-A specificities.

Only 3 of the 70 sera tested reacted by direct testing with normal lymphocytes, HeLa, HEp, and the 3 normal skin fibroblast cell lines. So this is an addition to ongoing specificity studies.

Of 120 patients with melanoma who underwent immunotherapy, 34% have died from secondary melanomas, 43% still have the disease, and 23% are free of the disease. I submit the data only as a summary. The patients (now totaling 140) are in different stages of immunotherapy, and this is not designed to indicate the entire series.

In conclusion, we are trying to study by more than one technique the humoral immunity in vitro and the cellular immunity. We are looking in vivo at skin test responsiveness and are also studying T- and B-cell response. It's quite interesting that these patients have demonstrated, upon completion of the immunotherapy program, >70% T-cells circulating; we are evaluating T- and B-cell interaction and the function of T-cells during each stage of immunotherapy.

We are looking at just a compilation of our data. You all can look at it. We are not interpreting these data because I don't believe we can at this time.

If I had to say, after looking at 140 patients with melanoma, which patients are likely to be helped—as Dr. Rapp indicated on the first day in a different system—in our experience we are helping those patients with cutaneous, subcutaneous, and nodal disease. If the patients have visceral, bone, or intracerebral diseases, we have not been able to help them.

Leventhal: Dr. Seigler, did any of your specific tests correlate with prognosis?

Seigler: The tumor-specific antibody in the patient's sera could not be correlated with the clinical stage in the first 70 patients studied. In other words, we had patients who did very well who had tumor-specific antibody, but we also had patients who had a continuation of their disease who had demonstrable antibody in their serum.

The 4 stages of immunotherapy do increase the antimelanoma antibody titer, but they don't seem to correlate with the clinical course.

As to the cellular immunity, we now can fairly regularly produce cell responsiveness in culture. We also can make patients who had no cell-mediated cytolysis demonstrate cell-mediated cytolysis by the regimen given. And in this population of patients, it can be correlated with what happens to them.

But the series is a bit selective in that, if you get to Stage IV—if you live that 8 months—obviously you are doing better with your tumor, anyway. So I don't know what to make out of that.

Now, our results differ a bit from some of the results presented this morning, in that we do have patients who have gone through all 4 stages who have demonstrable serum-blocking factor; we are studying that by the inhibition of the cell-mediated cytolysis and also in another system. We do have patients who do very well with serum-blocking factors who will block the cell-mediated cytolysis and who are doing fine.

We have some in vitro data suggesting that the blocking factor is really not greatly different from the antibody that is cytotoxic in the presence of rabbit complement. If you look at this in vivo, what it is doing, we just don't know. And of course the people studying the HL-A system have demonstrated the same thing.

In connection with Dr. Waksman's comments yesterday about cross-reactivity, one other interesting thing in these patients was the high degree of a newly developed heterophil antibody; you may miss some of the tumor-specific antibody if you don't absorb the serum with sheep red blood cells.

So I don't know what you are going to make out of it. But if you are absorbing the serum and you're not getting positive results, absorb the serum with sheep red blood cells. We've had several of the results come up positive when we did this.

Leventhal: I'd like to emphasize at least two of the points you made. First of all, let's not forget clinical staging as we do immune staging in these patients. And secondly, we've forgotten about cytotoxic antibody. We've been talking about antibody as though it were bad all day, and we should remember that it can kill cells.

Are there specific questions for Dr. Seigler?

Alexander: Dr. Seigler, when you do the mixed cell culture between the autologous lymphocytes and the autologous tumor cells, do you always treat the melanoma cells with neuraminidase, or do you get that reaction without neuraminidase?

Seigler: We don't get the reaction to work as well if the cells are not treated with neuraminidase. We can get some blastogenesis without neuraminidase, but it is very low.

Alexander: That has been our experience, but the procedure then gets a little worrisome because, if you now mix autologous lymphocytes and treat only half of them with neuraminidase, then of course you get stimulation in that situation too. So there is some danger that this has nothing to do with any new antigen on the melanoma cells.

Seigler: Even when we sometimes didn't get good lymphocyte stimulation in the autologous system, if we took the tumor cell, X-irradiated it, then added it to the lymphocyte as a responder, let this go on for 3–5 days—you can do either—then took this lymphocyte ("may be sensitized?"), placed it into the system of plus tumor cell that had been labeled, we could then see if it would lyse over and above that one exposed by this system. So we are measuring it that way also. I can't have much faith in the data yet because we have been doing this study for only a few months. But this population of cells so far shows a greater efficiency at lysis than the one that hadn't gone through this procedure.

Alexander: But the basic fact is that, in the non-neuraminidase-treated or autologous tumor cells, you get very little transformation. This has been our experience, too.

Gutterman: Dr. Seigler, how viable are those tumor cells that you use for stimulators?

Seigler: We don't use them if they are $<80\%$ viable.

Gutterman: Melanoma?

Seigler: Yes.

Gutterman: How do you separate the viable cells?

Seigler: We'll take the lesion off and then tease it out. And when I say "viable," I really have only two ways of thinking they're viable: 1) They exclude trypan blue—and I agree that's a very poor way, but anyway that's the way we look at it—and 2) the culture that we do from the rest of the tumor we don't use for stimulation. So those are two very inexact ways of looking at it, but at least that's how we do it.

Nathanson: Dr. Seigler, how long after you put your tumor cells down do you use them for your lymphocyte toxicity study?

Seigler: This varies. We test the monolayer to make certain that melanoma antigen is present before we set up the in vitro cytolysis. We have made now an anti-melanoma antibody in a heterologus system. This antibody has been absorbed with a variety of tumors, including lymphocytes, to remove all HL-A activity and, as far as we know, any cross-reacting tumor. This antibody has a good high-titer activity against all melanoma cells that we've looked at in the fresh situation. So we'll monitor our monolayer in that way.

And as I said, we're doing it now routinely in <9 passages, and we try to do it in passages 2, 3, and 4, because almost all of those have demonstrated what we think is melanoma-specific. . . .

Gutterman: What proportion of your cells can you get into culture at all for that 2 or 3 weeks after you've done your specific serologic testing?

Seigler: We've had about 50–50, which is not as good as what has been reported from most other laboratories. I don't know if we're just not as good, or what. We have to biopsy constantly.

T. Borsos: I would like to pursue your question a little further and ask Dr. Seigler, or anyone else in this room, two questions:

The first question is: Did any one of these monitoring techniques help to devise, revise, or improve the immunotherapy in the patients? Because that's obviously what one would like to use these techniques for. It's nice to know if the patient doesn't behave, but does it help you, the physician, to improve your techniques of treating him?

The second question, which is much more general is: Does anyone have any data, the sort of data Dr. Seigler presented to us, on patients who come into a clinic and are vigorously tested before anything else is done to them? And, if so, is there anything in these patients which makes them different from the normal population at that time?

Seigler: In our series, I don't think we can say that there is any in vitro technique which will tip us off that a certain patient will do well. That is why we continue all of this laborious work, because no one in the world would be happier than I and my technicians if someone would say that cellular immunity is measured by ^{125}I labeling. This is a lot of work, but it has to be done because we've got to study the mechanism.

Pinsky: In vivo skin testing certainly suggests when the patient first presents with his tumor, something about his prognosis depending on his skin test reactivity.

Borsos: How hard are these data, how were the patients behaving, and how sick were they? If the patients come in with a tiny tumor, like a primary tumor of the cervix, which is easily identifiable and eradicable, are they different from the normal population? I am fully aware of those studies which have been done on a few patients, but the patients, as far as I am aware, had very advanced diseases. Of course, you find impairment in them.

Seigler: What you just said is what we have observed. We have to look at our exceptions, and we have patients who come in with a small lesion or two that our skin test to those antigens right there will pick up delayed hypersensitivity in $>98\%$ of the population. And they will not react to a single one, and they'll do fine. And we, who are routinely in the transplant business, when we tried to recognize the anergic patient, couldn't do it. Right now we have no way of answering that question, and I think it's a good one.

Leventhal: There's a more important question here, too. I would guess that if the series of patients is large enough, you are going to find some correlation between skin test reactivity and size of tumor, because tumors are immunosuppressive. But there are about 3 factors that are going to go into this result: Is it the tumor that is immunosuppressing? Is it the fact that the patient was immunoincompetent that allowed the tumor to get that big? Or is it both together—which I guess most of us think it is?

So I imagine that the general correlation would have to hold that immunocompetent patients in general are going to do better.

Borsos: Don't you think it would be better to have an hypothesis and do the testing without thinking this way? Let us just test them and find out. I don't like this kind of biased thinking. That's because you have made up your mind that you are going to find it, and you are going to find it. If the person who reads it is going to do the test, you are going to find what you look for. I'm not accusing you of dishonesty, but we all have this tendency: We want to find what we're looking for.

Seigler: The same thing is true of the blocking factor. We want to say, "All right, all those patients who have blocking factor are those who don't do well." And that is just not so, and what's blocking factor in vivo?

Borsos: I fully agree that these tests ought to be done. But isn't it true that your only guidance—and everyone else's in this room—is how well does the patient handle his tumor, and that's what causes you then to decide what to do with the patient, and disregard almost anything else that you get from him? Is that what you're saying?

Seigler: That's correct. We devised a plan, because of some of Dr. Rapp's and Dr. Simmons' work; we decided to stick to it for the reasons I gave you, but the rest of it is a collection of data. The patients are going to go through Stages I to IV if they live, whether I like it, or they like it, or not. The rest is just compilation, and then we would like to look and see what does, if anything, correlate with anything.

Borsos: What happens if it doesn't correlate with anything? Are you going to stop immunotherapy? Probably not. You will continue to look for the patient's improvement. Therefore, these kinds of discouraging results should not discourage us from doing therapy in patients.

Leventhal: We do need the data in animals, too, Dr. Borsos. That's the point that Dr. Herberman made: We really do need to know, and it's something that could be worked on in an animal model as well.

Borsos: It is being worked on in the animal models. A great deal of data are being collected.

Herberman: I wanted to respond to what Dr. Alexander said. First of all, in terms of radiated cells giving a reaction or not, one possibility, of course, is that the radiation itself could hurt the antigens. Secondly, Dr. Santos has been testing with intact cells. We made membrane extracts for him which he also tested. So far, the correlation of results with radiated cells and membrane extracts has been excellent in a few tests.

Alexander: This is in acute leukemias?

Herberman: Yes.

Santos: Primarily AML.

Herberman: We have also tested 5 patients of our own with whole cells as well as with the membrane extracts, and the correlation also has been excellent.

As to the question of bacterial contamination, if it's an artifact, it's a tumor-associated artifact because we had the controls of making the very same type of preparation from remission material and we haven't seen reactivity with that.

Secondly, we monitored our testing bacteriologically at all stages. We can certainly draw blood sterilely. You can easily do the entire procedure sterilely. So it's very difficult for me to see how the bacteria are getting in there to produce that artifact.

Gutterman: Dr. Santos, with what dose of tumor cells are you getting positive tests?

Santos: With the whole cells, there's 25 million—this is remission—and then we've done some with the 25 million lymphocytes. They may not be the best controls.

Gutterman: I guess the AML cells in Texas resemble those in England because we've tested the last patients now with AML with whole cells, ranging from as little as 10,000 cells to as high as 100 million.

Alexander: Irradiated?

Gutterman: Yes. And we have never seen a positive skin test. In contrast, as some of you know, we have been seeing positive skin tests, perhaps even delayed hypersensitivity, with the KCl-extracted antigen and, at the same time, have been seeing negative skin tests with the whole cells.

Mardiney: Dr. Santos, is the dose of radiation the same in all cases, as well as Professor Alexander's? That may be an important point. What dose of radiation were you giving these cells?

Santos: We gave them 10,000 R, but I ought to add this is 3 of 5, and maybe the next 100 are going to be negative.

Seigler: This isn't exactly skin testing but it has something to do with the antigen: We have taken blood from patients with both lymphatic-derived leukemias and myelogenous-derived leukemias and placed it in the blood cell separator; blood from the same patients was used to immunize the same monkey and was then specifically absorbed, giving an antibody that reacted specifically with either lymphogenous or myelogenous cells by cytotoxicity.

So there is an antigen present on the surface of these cells. It is probably tumor specific, and even to the degree of one derivation versus the other. This experiment is to be published by J. L. Miller and R. S. Metzgar.

Alexander: The mystery of it is not that there isn't an antigen—there's lots of evidence—but we have the same problem even with the ordinary HL-A antigens. After all, if you do ordinary leukocyte injections in sensitized people, you do not get skin reactions.

Seigler: You'll get skin reactions if you have an allogeneic system. There will be some basis for the old third-man test.

Leventhal: I'd like to move on to another topic, since it's clear we are not going to settle this with excellent but conflicting data. It's healthy for us to know that the question isn't settled, and I don't see a solution looming. If someone has the answer, he or she is welcome to give it.

I'd like to call on Dr. Chretien, who has just one more assay—in vivo assay—that might be correlated with response of patients to therapy.

Quantitative Dinitrochlorobenzene Contact Sensitivity in Preoperative and Cured Cancer Patients[1]

Paul B. Chretien, Patrick L. Twomey, Emile E. Trahan, and William J. Catalona, *Surgery Branch, National Cancer Institute,[2] Bethesda, Maryland 20014*

SUMMARY—With a technique that quantitates dinitrochlorobenzene (DNCB) contact sensitivity, the DNCB reactivity of 201 preoperative cancer patients was compared with that of 73 patients who had sufficiently long intervals after resection of malignancies to be considered cured. Controls were 143 healthy volunteers. The preoperative cancer patients had high incidences of anergy (22% vs. 2.8% in controls) and impaired positive reactivity (23% vs. 1% in controls). The incidences of abnormalities were not significantly influenced by tumor histology or extent of tumor growth. Among cancer patients considered cured after previous resection of sarcomas, melanomas, and adenocarcinomas, DNCB reactivity was normal; but patients previously treated for squamous carcinomas of the head and neck and pelvic organs had the high incidences of abnormal DNCB reactivity seen in preoperative patients. The results indicate that quantitative DNCB reactivity is useful in monitoring the response to cancer therapy, but tumor histology influences the interpretation of abnormal reactivity in treated patients.—Natl Cancer Inst Monogr 39: 263–266, 1973.

INCREASING EVIDENCE indicates an association between the cellular immunologic reactivity of cancer patients and their response to therapy (*1, 2*). These studies emphasize the need for quantitative assays of cellular immunity that correlate with the clinical status of cancer patients.

The demonstrations that anergy to dinitrochlorobenzene (DNCB) correlates with a poor response to both surgical (*1*) and immune therapy (*3*) in patients with nonlymphoid tumors prompted the development of a quantitative assay of DNCB reactivity (*4*). Studies in normal persons (*5*) and cancer patients (*6*) showed that this assay technique delineated at least 3 levels of DNCB sensitivity and revealed a previously unappreciated high incidence of impaired DNCB reactivities in patients with a variety of clinically operable solid tumors. Assay of the reactivity of patients, with a sufficiently long interval after surgical excision of malignancy to be considered cured, shows correlations with clinical status and DNCB reactivity that further establishes the usefulness of this technique. It also reveals, however, a limitation of DNCB reactiv-

[1] Presented at Conference on the Use of BCG in Therapy of Cancer, National Institutes of Health, Bethesda, Md., Oct. 5 and 6, 1972.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

ity as a predictor of clinical status of treated patients that is related to the histologic type of tumor previously resected.

MATERIALS AND METHODS

Preoperative patients.—Studied were 201 patients admitted to the Surgery Branch, National Cancer Institute, for definitive surgical resection of malignancies. Only patients with clinically operable tumors who were in good general health were included. In some patients, metastases were found during preoperative evaluation or during surgical exploration. Patients who had undergone surgery, chemotherapy, or radiation therapy within the previous 3 months or those with chronic disease, drug ingestion, or active infections were excluded. They were divided into 5 groups according to the histologic type of tumor present: bone and soft tissue sarcomas (44 patients); melanomas (41 patients); adenocarcinomas (31 patients); head and neck squamous carcinomas (44 patients); and pelvic squamous carcinomas (41 patients).

Cured patients.—Studied were 73 patients who had been treated for histologically proved malignancies at the Surgery Branch, National Cancer Institute. All patients were in good general health at the time of the study. Each had undergone a physical examination within 3 months of the study and were considered clinically cured of tumor at that time. Only patients with a normal hematocrit, white blood cell count, sedimentation rate, and other appropriate laboratory studies were included. Patients who had undergone postoperative chemotherapy, radiation therapy, or immunotherapy and those taking medications known to alter immune reactivity were excluded.

Like the preoperative patients, these patients were divided into 5 groups according to the histologic type of tumor excised. 1) Sarcomas (11 patients): Three patients had osteosarcomas, and eight had soft tissue sarcomas. All had been treated by radical excision of the primary tumor. Their disease-free interval was 5–11 years (median 7 yr). 2) Melanomas (9 patients): These patients had been treated by wide excision of the primary site and regional node dissection. Their disease-free interval was 6–14 years (median 8 yr). 3) Adenocarcinomas (15 patients): Nine patients had adenocarcinomas of the breast, and six had adenocarcinomas of the large intestine. All had been treated by standard surgical resection. Their disease-free interval was 5–12 years (median 9 yr). 4) Head and neck squamous carcinoma (21 patients): These patients had been treated by excision of the primary tumor and, in most instances, by radical neck dissection. Although most had been heavy smokers preoperatively, only 7 admitted to tobacco use in any form when studied. Patients with a history or other evidence of alcohol abuse postoperatively were also excluded. Their disease-free interval was 4–13 years (median 9 yr). 5) Pelvic squamous carcinoma (22 patients): Nineteen patients had primary cervical carcinoma, and two had primary vulvar carcinoma. Nine cervical cancer patients had been treated by radical hysterectomy and the remainder by total pelvic exenteration. Patients with chronic urinary tract infections were excluded. Their disease-free interval was 7–15 years (median 9 yr).

Control subjects.—Controls were 143 healthy volunteers solicited from the employees of the National Institutes of Health and from residents of a retirement community. Their ages ranged from 20 to 80 years and were almost equally distributed in each decade represented. Controls were screened to exclude those who had chronic disease, were taking any medications, or had a recent illness or history of malignant disease.

Quantitation of DNCB contact sensitivity.—The method for quantitating DNCB reactivity (*4*) incorporates the spontaneous flare reaction which occurs at the site at which DNSB is applied 7–14 days beforehand. Doses of 2000 μg and 50 μg of DNCB were applied to the upper arm and forearm, respectively. A spontaneous flare at both the 2000-μg and 50-μg challenge sites by 14 days was scored 4 +. A spontaneous flare at only the 2000-μg challenge site was scored 3 +. If a spontaneous flare did not develop by 14 days after application of DNCB in either site, 50 μg was applied to the opposite forearm. If a delayed cutaneous hypersensitivity reaction (DCH) occurred at this site within 48 hours, it was scored 2 +. An equivocal reaction which required biopsy for confirmation was scored 1 + if the histologic features of DCH were present. If these changes were not seen or if no gross reaction occurred, the subject was scored anergic to DNCB.

Statistical methods.—Comparisons of DNCB reactivity of the patient groups with each other and the normal population were done by chi-square analysis.

RESULTS

DNCB Reactivity of Normal Controls

A spontaneous flare reaction developed within 14 days of DNCB sensitization in 138 (96.5%) of 143 controls. Of the 138, 39% had 4 + reactions and 57% had 3 + reactions. When challenged with 50 μg of DNCB, 1 of the 5 subjects who failed to develop a spontaneous flare manifested a 2 + reaction. The remaining 4 (2.8%) were anergic; they were 42, 58, 62, and 80 years old. There was no significant correlation between age and the incidence of 4 + and 3 + reactions or anergy (table 1).

DNCB Reactivity of Preoperative Cancer Patients

As a group, the DNCB reactivity of preoperative cancer patients was abnormal when compared with that of the controls. Only 55% of

TABLE 1.—Comparison of quantitative DNCB reactivity of preoperative and clinically cured cancer patients with normal controls

Patient group	Status	DNCB reactivity				
		Number of patients	Percent anergic	Percent impaired positive (1–2+)	Percent normal (3–4+)	Statistical difference from normal controls*
Normal controls	—	143	3	1	96	—
Sarcoma	Preoperative	44	19	19	64	$P < 0.01$
	Cured	11	0	0	100	†
Melanoma	Preoperative	41	12	29	59	$P < 0.02$
	Cured	9	11	0	89	†
Adenocarcinoma	Preoperative	31	24	39	49	$P < 0.01$
	Cured	15	7	7	87	†
Head and neck squamous carcinoma	Preoperative	44	27	14	59	$P < 0.01$
	Cured	16	12	12	75	$P < 0.01$
Pelvic squamous carcinoma	Preoperative	41	44	23	32	$P < 0.01$
	Cured	22	27	5	68	$P < 0.01$

* By chi-square analysis.
† Not statistically significant.

preoperative cancer patients developed spontaneous flare reactions to DNCB, an incidence that was significantly less than that in controls ($P < 0.01$). After patients who failed to develop spontaneous flare reactions were challenged with 50 μg of DNCB, 23% developed 2 + or 1 + reactions and 22% were anergic. Furthermore, when grouped according to tumor histology, the distribution of reactivities of the patients in each category differed significantly from that of the normal controls ($P < 0.01$) (table 1). The patients in each histologic category were divided, according to the extent of tumor determined preoperatively and at operation, into those with localized tumors and those with metastases. In none of the histologic categories did the reactivities of the patients with localized tumors differ significantly from those with metastases.

DNCB Reactivity of Cured Cancer Patients

Paralleling the findings in preoperative cancer patients, the DNCB reactivity of cured cancer patients when considered as a group differed significantly from that of the controls. However, when tumor histology was considered, differences in the distributions of reactivities were demonstrated that correlated with this variable. Among patients who previously had resection of sarcomas, melanomas, and adenocarcinomas, the distribution of DNCB reactivities did not differ from that of the normal controls; by contrast, patients previously treated for squamous carcinomas of the head and neck region and pelvic organs had incidences of anergy and impaired positive reactivity that rendered the distribution of both groups significantly abnormal when compared with that of the controls ($P < 0.01$). These incidences of abnormal DNCB reactivity also parallel those seen in preoperative patients who had tumors of this histologic type (table 1).

DISCUSSION

The results obtained with the relatively large number of patients studied with quantitative DNCB reactivity in this report substantiate the usefulness of this assay shown in preliminary studies (*4, 6*). The findings in preoperative patients with solid malignancies show that abnormalities of DNCB reactivity often occur early in the clinical evolution of the tumors. This minimizes the likelihood that the abnormalities seen are nonspecific and secondary to advanced disease. Furthermore, with each histologic type of tumor, the use of quantitative DNCB reactivity

significantly increased the proportion of abnormalities demonstrated when compared with that which would have been revealed by qualitative assay of DNCB reactivity.

Among the cured patients, the distribution of DNCB reactivity of those who had previously undergone resection of sarcomas, melanomas, and adenocarcinomas did not differ from that of normal persons. This finding provokes the speculation of whether the abnormalities seen in the preoperative patients with tumors of these histologic types revert to normal after treatment, or if patients with these tumors who have abnormal DNCB reactivity are not cured despite treatment. Determination of the clinical course of the preoperative patients studied will eventually resolve this point. At present, however, it appears that patients previously treated for malignancies of these histologic types, who have had sufficiently long disease-free intervals to be considered cured and yet display abnormal DNCB reactivity by this assay technique, may be suspect of having residual tumor. The utilization of the assay to give insight into the status of treated cancer patients, however, will require study of large numbers of patients clinically cured of malignancies of these histologic types and long-term observation of the course of those patients with abnormal DNCB reactivity.

The findings in clinically cured patients previously treated for squamous carcinoma contrast sharply with the normal reactivity of patients who had tumors of other histologic types, since the incidences of abnormalities were similar to those found in preoperative patients with squamous carcinoma. These persisting immune defects may have important implications for investigations of the etiology and pathogenesis of squamous carcinoma. The mounting evidence for an association between herpes simplex viruses and the development of squamous carcinomas (*7*) and the demonstrated in vitro immunosuppressive effect of viral infections (*8*) should lead to investigations to determine if these agents cause the persisting defects. Another possibility to be explored is that patients who develop squamous carcinoma constitute a cohort of the population who have genetically determined impaired cellular immunity that predisposes them to develop squamous carcinoma. The persisting defects in these patients also may have relevance to the selection of therapeutic modalities, particularly in the consideration of therapies which may suppress immune reactivity.

These comparisons of the quantitative DNCB reactivity of clinically cured and preoperative cancer patients thus give a unique insight into aspects of the host-tumor relationship not previously appreciated and reveal both an asset of this assay as a monitor of clinical course of cancer patients and a limitation of this assay in relation to tumor histology.

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DISCUSSION

B. G. Leventhal: Rather than just giving an all-or-none phenomenon, this gets into how some of these reactions could be better quantitated. Perhaps this will help us to clear up some of these areas of confusion.

C. M. Pinsky: We have obtained similar data using the dinitrochlorobenzene (DNCB) skin test.[1] We also have seen a decreased incidence of DNCB reactivity in patients with squamous cell carcinoma.

In addition, and particularly to answer the question raised earlier by Dr. Borsos, we have confirmed the findings of Eilber and Morton[2] that DNCB reactivity at the time the patient is first seen correlates extremely well with short-term prognosis after definitive cancer surgery ($P < 0.0005$ and < 0.001).

It is interesting—and Dr. Sokal made this point several months ago—that reactivity to this antigen, apparently unrelated to tumor antigens, correlates better with the course of the cancer than any in vivo or in vitro reactivity to presumed tumor antigens yet reported.

Leventhal: I suggest that one reason for this is that we know more about the nonspecific tests, and we still do not know what the best type of preparation is to use to test for specific tumor immunity. Did you want to comment about skin testing, Dr. Alexander?

P. Alexander: Dr. Pinsky and Dr. Morton have seen a correlation between prognosis and the failure of the patient to mount a good delayed-hypersensitivity response. Possibly these 2 parameters are only indirectly connected. Very likely, a direct correlation exists between extent of disease and failure to mount a delayed-hypersensitivity reaction. Hence, delayed hypersensitivity may only be a rather clumsy way of expressing the amount of tumor in the patient. It is hardly surprising that those patients with a lot of tumor generally tend to do worse than those with a little tumor. In other words, the immunologic depletion results from the disease, and the best way of dealing with it is to reduce the amount of disease.

Pinsky: Dr. Alexander, no one doubts that innumerable factors may play a role in determining a skin test result at any point in time. But, for the patient, at the time of early diagnosis—and this is the patient about whom Dr. Borsos is asking—reactivity to DNCB does imply a better prognosis, which makes the test useful whatever the mechanism may be.

Leventhal: Does anyone have a macrophage assay to describe? Mechanisms by which the immune system can kill tumor cells include antibody and killer T-cells and B-cells, as Dr. Waksman mentioned, but they also include macrophages, which indeed shouldn't be omitted. I have heard no discussion about assaying the activity of macrophages either in man or animals.

G. W. Santos: This method is just being developed sort of conceptually, but one could put the migration-inhibition factor (MIF) in the skin. The trouble with that is that it is too hard to generate it. A factor from the starfish that R. A. Prendergast has described at least on one testing can be given and has behavioral properties very much like MIF. It turns on macrophages, increases their lysosomal activity, etc., inhibits their migration. If put in the skin of a variety of species, it in fact gives you something that looks identical under the microscope in a short time to delayed hypersensitivity.

It's at least one test, because we found an individual in another situation who was completely negative to skin tests who would not react to material to which the guinea pig reacted in his own skin; it is as if he could not react to this material. But that's the only sort of macrophage.

Leventhal: It's a good suggestion. Dr. Rosenthal, did you have an assay?

S. R. Rosenthal: A very neat test (easy to do and very clear-cut) is G. Barksi's macrophage test for an animal model. I saw it in operation. Dr. Barski takes the macrophages from the abdomen of a mouse; he is very careful not to stimulate it with oil; he washes the peritoneum, he says that's very important, with a physiologic solution. He obtains the macrophages, mixes them with the tumor cells of a homologous mouse, and finds that growth of these tumor cells is inhibited as long as the tumor, as I already mentioned, is a certain size, i.e., up to about 1 cm. When the tumor becomes larger, macrophages do not inhibit tumor cells in vitro. If the tumor is removed, the cytotoxic effect of the macrophages on tumor cells returns in 2 months.

T. Borsos: D. A. Boetcher in our laboratory is testing the chemotactic response of macropbages with a test developed by R. Snyderman who is now at Duke University. This test measures the ability of macrophages to go through a porous membrane. It is now being used at Duke University and in our laboratory in animal and human models to look for possible deficiency of macrophages from cancer patients.

Leventhal: So at next year's conference we will hear about that.

Rosenthal: Some experiments on severe burns may be operative in chemotherapy. After burning, the immune system is greatly depressed both by skin and in vitro testing. In extensive, severe burns, antibiotics are ineffective even though the organisms isolated from the wound or blood are sensitive to antibiotics. I postulate that, for antibiotics to be effective, the immune system must be intact. A similar condition may exist in chemotherapy in oncology. Therefore, I recommend that BCG or other immunologic agents be given before, during, and after chemotherapy or surgery.

Leventhal: Dr. Hanna, did you want to comment?

M. G. Hanna, Jr.: I'd like to return to our earlier discussion about the relevance of animal models. In particular, I'd like to direct a comment to Dr. Mardiney. Dr.

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[1] Pinsky CM, Oettgen HF, El Domeiri A, et al: Delayed hypersensitivity reactions in patients with cancer. Proc Am Assoc Cancer Res 12:100, 1971.

[2] Eilber FR, Morton DL: Impaired immunologic reactivity and recurrence following cancer surgery. Cancer 25:362–367, 1970.

Mardiney pleaded for the AKR system and its relevance to the leukemia studies in man. Though this system does have some potential, I would like to provide a warning.

AKR mice have an abundance of endogenous leukemia virus. It has recently been shown that they have autogenous immunity to the virus which is expressed in detectable specific antibody not found circulating free, but found associated with the immune complex disease in the kidneys of these animals; this antibody can be eluted, at low pH from the kidneys, and can be specific for Gross virus-mediated, cell-surface antigens.[3]

Therefore, throughout their life, these animals have a chronic immune response to the leukemia virus. With the AKR strain, it doesn't seem to be beneficial, because these animals still have a high incidence of lymphoma. However, in other mouse strains, especially those with a low natural incidence of lymphomas, we have recently demonstrated an inverse correlation between the level of autogenous immunity and the lymphoma incidences.[4]

The point I'd like to make is: What you may be doing with BCG in the AKR mouse is modifying the quantity and the quality of the humoral immune response to the virus and actually affecting the etiologic agent of lymphomas in these animals, rather than doing something on the order of what's happening in human leukemias or in the guinea pig models, such as a BCG tuberculous response, resulting in tumor killing.

Do you have any evidence of this? Are you actually affecting cell-mediated immunity against virus-mediated, tumor-specific, cell-surface antigen?

M. R. Mardiney, Jr.: We don't have any evidence; we are working on obtaining such evidence.

The immune complex disease occurring in the AKR mouse is secondary to a poor antibody response. The AKR mouse has always exemplified the animal specifically tolerant to virus, because antibody to virus could never be detected. The AKR strain responds meagerly. Every time a molecule of antibody goes out to the periphery, it gets removed from the circulation because the antigen is in excess. So the AKR strain is a model in which responsiveness is poor. Can we make responsiveness better? We are looking at ways to measure that.

Leventhal: Dr. Stone, did you want to make a comment?

S. H. Stone: I just thought that the goal of any clinical investigator should be determined ahead of time whether he's really trying for the nonspecific response or for the specific one, because in each case his schedule of treatment should differ.

As Dr. Rosenthal pointed out, the amounts of BCG for the one greatly exceed the amounts you need for the other, so that if you are going for both, you'd be faced with a dilemma, how to set up your protocol. Thus we want to separate those two approaches before we select the quantities of BCG.

Leventhal: That's an excellent point, and I wanted to add to the point Dr. Weiss made yesterday that indeed we may want both. If we can't get stimulation of a specific antitumor response, we may still want general stimulation of immune response to give more chemotherapy. Now, I'd like to turn the meeting over to William Terry.

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SUMMARY REMARKS

Before I make my comments, I would like to point out that anyone who has ever had to organize a meeting involving participants from many countries knows of the tremendous amount of work necessary. A meeting proceeding as smoothly as this one has reflects the hard work on the part of Dr. Borsos, the people in his office, and his colleagues, whom we now thank.

One of the characteristics of this meeting, and perhaps one of its flaws, is that, since we have been given such a wide variety of information, approaches, and topics, it really hasn't been possible to dwell in sufficient detail on many of the areas warranting detailed discussion. The breadth of the meeting has been so great that it probably would not be useful to attempt to summarize all the scientific content.

Therefore, I shall try to make some generalizations that may express the sense of the meeting and make personal observations concerning the directions in which I see things moving.

It is quite clear that the general area of immunotherapy of cancer is one of bright promise. The specific approach to immunotherapy dealt with in this meeting, i.e., the use of BCG, has rapidly moved ahead in the past decade and has yielded some provocative results.

The heat of some of the exchanges between the participants reflects the healthy state of investment of ego that committed investigators have, which is to be expected in any rapidly expanding area in biomedical research.

As is usually true in rapidly developing research, there are a number of limited hard facts. These facts should be emphasized and used as a basis to think about future directions.

Fact number one, of fundamental importance, is that an established tumor can be destroyed in animals by some manipulation of the immune system, as demonstrated over the past 3 years by Dr. H. J. Rapp and his group. Were it not for the demonstration that immunomanipulation alone can indeed destroy a tumor, we would not be ready to take off in the way that we are.

In animals, not only is the tumor destroyed, but also the animal appears to be cured. We must clearly distinguish between destruction of local tumors and cure of the host, a point to which I will return later.

It must be emphasized that the cure is accomplished in a highly artificial system: one with many important differences from the circumstances existing in human clinical oncology. For example, the tumor is transplanted and is not spontaneous; it is a liver tumor but is transplanted into the skin. Moreover, there are severe limitations on the therapeutic effect because treatment is effective only when administered while the tumor is relatively small and appears quite ineffective when the tumor is located subcutaneously or intramuscularly rather than intradermally.

There are additional qualifications, but these must not obscure the fundamental observation that immunomanipulation alone can destroy the tumor and cure the animal.

Fact number two is that other forms of immunomanipulation applied in animals also give evidence that immunotherapy can destroy tumors and cure the animal. The work that Dr. R. L. Simmons has published and reviewed for us is another approach: By modifying the surface of the tumor cell in ways not yet completely understood, one can heighten the immune response against the tumor, which leads to the destruction of the established tumor and cure of a certain proportion of the animals.

As with the BCG studies, Dr. Simmons' experiments must be interpreted in the light of certain qualifications and limitations; but, again, the important point is that, at least

under certain circumstances, immunotherapy can permanently cure an established tumor.

Fact number three is that certain types of human tumors fairly regularly can be destroyed by local immunomanipulation. The review presented by Dr. E. Klein and some of the reports on intralesional injection of BCG into melanoma lesions have clearly established that, using the kind of manipulations described, one can indeed destroy certain accessible human tumors.

Having said that some local tumors can be destroyed, I must point out that there is little convincing evidence at this time that any patients have been cured by immunotherapy alone or by the addition of immunotherapy to other forms of cancer treatment. This evidence will be eagerly sought during the coming years; this search will require carefully controlled clinical trials to establish the usefulness of immunotherapy.

The work of Dr. Klein and others has shown that local collections of a number of tumors can be destroyed by a variety of means in man. Having established this possibility, one must define the conditions under which this treatment can or should be used. Appropriate clinical trials must be conducted to determine whether this form of therapy has any advantages over present modes of therapy and whether the former can be combined with the latter to give better results.

The other type of clinical trial with which we will be concerned in the next decade involves the potential applicability of immunotherapy to inaccessible malignancies. We then will be confronted with difficult questions concerning patient selection, study design, avoidance of the possibility of facilitating tumor growth in some patients, and the ethicalness of substituting new treatments of unknown efficacy in patients for established treatments of at least limited benefit. These questions will be answered only through the hard work of many clinicians and laboratory investigators, and it will take many years before we have information that may be of use in the routine treatment of patients with cancer.

The papers presented today have given some encouraging results concerning the feasibility of carrying out appropriate studies. The carefully conceived and controlled studies reported on by Dr. R. Powles provide a model; when this study is completed, there will be interpretable and useful data.

These studies do not give us the kind of anecdotal information that we obtained this morning, nor do they give us data that are immediately interpretable. These studies are frustrating because it takes many years of back-breaking work in the clinic to carry them out and to go through the rigorous procedures involved in a controlled clinical trial. But without these studies, and without the information they will provide, we will never answer any of the questions concerning the clinical application of immunotherapy.

As we proceed with immunotherapy, we must constantly remind ourselves that cancer is not a single entity. We cannot fall into the trap of assuming that something which looks promising in the therapy of melanoma has necessarily anything to do with the therapy of prostatic cancer. There are many different cancers, each having its own special biologic potentials and peculiarities, its own ways of metastasizing, and its own peculiar consequences. As long as we are dealing with relatively empiric approaches in immunotherapy, we must take the empiric road in finding appropriate therapy for each type of cancer. It probably will not be possible to reach broad generalizations about immunotherapy of cancer. Instead we will have to deal with immunotherapy of particular malignancies.

It is worth raising the question of whether, if we can learn something about mechanisms in the immune system as they apply to reaction against tumors, it will be possible to design more rational approaches to immunotherapy.

Playing the devil's advocate for a moment, I would like to point out that the history of medicine flies in the face of that expectation. Most treatments or cures that have had significant impact in human medicine really were discovered and applied long before fundamental mechanisms were understood. It is certainly possible that the same will be true for immunotherapy and that if effective immunotherapy is developed, it may well occur before we understand the nature of the immune processes involved.

This meeting has been directed toward discussions of BCG and immunotherapy. However, there are obviously other approaches to immunotherapy and other ways of manipulating or utilizing the immune system.

To effectively manipulate and use the immune system, we need to understand as fully as possible the nature of this system. As a side issue, I will take you back to the colloquy between Dr. T. Borsos and Dr. B. H. Waksman yesterday afternoon, with the benefit of a day's interval between.

Clearly, if we do not study cancer, we will not learn anything about cancer. Obviously, if we really wish to learn about mechanisms in cancer, we will have to deal with tumor systems.

However, it is equally important to accept and understand that our capacity to apply immune mechanisms to tumors will greatly depend on information coming from studies that have nothing to do with tumor systems; i.e., our colleagues who study the fundamental mechanisms in cellular immunology, humoral immunology, and genetic control of immune responses will provide us with the very information and tools required and very much needed.

There is clearly not a need for either/or; rather there is a need for both. We must not only look at tumor systems, which is absolutely mandatory, but also learn as much as we can about the fundamental mechanisms in the immune system of animals and man.

There was a discussion this afternoon about in vitro testing, an area which is quite unclear to us. We can only hope that continual searching in the area of in vitro tests or in vivo tests will supply us with the kind of needed information which will be useful in selecting out those patients who will be most appropriate for possible immunotherapeutic maneuvers in the future or for following these patients and helping us to monitor and guide their therapy as it proceeds.

Although work has been going on for quite some time now, we are just at the beginning of the story of BCG and immunotherapy. BCG is obviously a relatively crude material, with all due respects to those of you who produce and work with it. Our applications with BCG are really not too distant from the kind of folk medicine alluded to yesterday by Dr. J. L. Ziegler, who very appropriately pointed out that any good witch doctor will tell you that scarification should be applied over the lungs—even without any guinea pig experiments.

For all its primitive qualities, BCG is a beginning in immunotherapy. Clearly there will be improved products to use, whether it be the methanol-extraction residue or some other extract of BCG. We must identify the stimulatory agent within BCG and prepare it in such a form so that it will cause fewer side effects or complications.

Let me remind you of the comment Dr. H. J. Rapp made in relating the long-standing observations about spontaneous regressions of cancer in man being associated with infectious processes. Not all of these infections were tuberculous. Therefore, to limit ourselves to the use of BCG and related substances in our therapeutic approach would be foolish; we must broaden our horizons and look for other substances, bacterial or otherwise, which may also be less toxic and more effective in inducing the kinds of stimulations of the immune system in which we are interested.

Finally, we have to concern ourselves with the role immunotherapy will play vis-à-

vis the other forms of therapy that we lump together under the general designation of conventional therapy.

We tend to discuss this question as if we were dealing with mutually exclusive alternatives, which I believe is irrational and improper.

All of us must remember that our ultimate goal is either to find a means of preventing people from getting cancer or to cure established cancer. Most of us hope that immunotherapy will provide a new useful approach to achieving this goal. Possibly there are some cancers for which immunotherapy will be the primary therapy. It's equally likely that there will be other cancers for which immunotherapy will be useless. It seems probable that the most common situation, however, will be one in which multiple forms of therapy will be needed. The clinician will have to find means of judiciously balancing and utilizing radiotherapy, chemotherapy, surgery, and immunotherapy in a way that will be optimal for a particular patient with a particular neoplasm.

Our goal is to be able to improve the life expectancy of the patient with cancer. Our job for the coming years, whether it be in immunotherapy or in other kinds of therapy, is to achieve that goal.

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